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Anticipated Classification of  
this Application:

Class: Subclass:

For Application:

Examiner: M. Pak

Art Unit: 1812

Box PATENT APPLICATION FEE

Assistant Commissioner for Patents  
Washington, DC 20231

Sir:

This is a request for filing an

☐ Original

☒ Continuation

☐ Divisional

☐ Continuation-in-part

application under 37 C.F.R. 1.53(b), in the name of Henry A. Lester; Norman Davidson; Paulo Kofuji (Names of ALL Applicants), for INWARD RECTIFIER, G-PROTEIN ACTIVATED, MAMMALIAN, POTASSIUM CHANNELS AND USES THEREOF (Title of Invention). This ☒ continuation ☐ divisional ☐ continuation-in-part claims priority to pending application Serial No. 08/614,801, filed on March 7, 1996.

1. (a) ☐ Enclosed is a new application.
- (b) ☐ Enclosed is a continuation-in-part application.
- (c) ☒ Enclosed is a copy of the prior application.

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DATE OF DEPOSIT March 16, 1998

I HEREBY CERTIFY THAT THIS PAPER OR FEE IS BEING DEPOSITED WITH THE UNITED STATES POSTAL SERVICE "EXPRESS MAIL POST OFFICE TO ADDRESSEE" SERVICE UNDER 37 CFR 1.10 ON THE DATE INDICATED ABOVE AND IS ADDRESSED TO: BOX PATENT APPLICATION FEE, ASSISTANT COMMISSIONER FOR PATENTS, WASHINGTON, DC 20231.

TYPED NAME Lisa Leos

SIGNED *Lisa Leos*

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Form 1.16b (8069)

01/98

2. (a) ☐ Enclosed is a new Declaration.  
 (b) ☒ Enclosed is a copy of the prior executed Declaration as originally filed.  
 (c) ☐ Enclosed is a Combined Declaration/Power of Attorney.
3. (a) ☐ Enclosed is a Small Entity Affidavit.  
 (b) ☒ A Small Entity Affidavit is of record in the prior application.
4. ☒ The filing fee is calculated below:

Claims as filed in the prior application, less any claims canceled by amendment below:

	(Col. 1)	(Col. 2)	SMALL ENTITY			OTHER THAN A	
FOR:	NO. FILED	NO. EXTRA	RATE	FEE	OR	RATE	FEE
BASIC FEE				\$395	OR		\$790
TOTAL CLAIMS	<u>17</u> - 20 =	* <u>0</u>	x11 =	\$	OR	x22 =	\$
INDEP CLAIMS	<u>4</u> - 3 =	* <u>1</u>	x41 =	\$ <u>41</u>	OR	x82 =	\$
[ ] MULTIPLE DEPENDENT CLAIM PRESENTED			+135 =	\$	OR	+270 =	\$
*If the difference in Col. 1 is less than zero, enter "0" in Col. 2.			TOTAL	\$ <u>436</u>	OR TOTAL		\$

5. ☒ The Filing Fee is not enclosed.
6. ☐ Our check in the amount of \$\_\_\_\_\_ is enclosed.
7. ☐ Cancel in this application original claims \_\_\_\_\_ of the prior application before calculating the filing fee. (At least one independent claim must be retained for filing purposes.)
8. ☒ Amend the specification by inserting before the first line the sentence:

--This is a ☒ continuation \_\_\_\_\_ division \_\_\_\_\_ continuation-in-part of application Serial No. 08/614,801 filed March 7, 1996.--

9. (a)   X   Informal drawings are enclosed,   6   sheets.  
(b)        Formal drawings are enclosed,        sheets.
10. (a)        Priority of application Serial No.                    filed on         
           in    is claimed under 35  
U.S.C. 119.
- (b)        The certified copy has been filed in prior application  
Serial No.                    filed on                   .
11.        An Assignment is enclosed.
12.   X   The prior application is assigned of record to California  
Institute of Technology.
13.        A Power of Attorney by Assignee is enclosed.
14.   X   The power of attorney in the prior application is to:  
  
FLEHR HOHBACH TEST ALBRITTON & HERBERT LLP  
4 Embarcadero Center, Suite 3400  
San Francisco, California 94111
- (a)   X   The power appears in the original papers in the prior  
application.
- (b)        Since the power does not appear in the original papers, a copy  
of the power in the prior application is enclosed.
- (c)   X   Address all future communications to:

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Suite 3400, Four Embarcadero Center  
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15. \_\_\_\_\_ A preliminary amendment is enclosed. (Claims added by this amendment have been properly numbered consecutively beginning with the number next following the highest numbered original claim in the prior application.)

16. \_\_\_\_\_ A Prior Art Statement is enclosed.

17.      X      I hereby verify that the attached papers are a true duplicate of prior application Serial No. 08/614,801 as originally filed on March 7, 1996.

Date: 3-14-98 Dall Jr

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## INWARD RECTIFIER, G-PROTEIN ACTIVATED, MAMMALIAN, POTASSIUM CHANNELS AND USES THEREOF

The invention disclosed herein was made with U.S. Government support  
10 under USPHS grants GM29836 and MH49176. The U.S. government has  
certain rights in this invention.

### CROSS-REFERENCE TO RELATED APPLICATIONS

This application is a continuation-in-part of U.S. Patent Application Serial  
15 no. 08/066,371, filed March 21, 1993.

### INTRODUCTION

#### Background

The regulation of heart rate in response to stress, activity, and other  
20 stimuli is essential for mammalian survival. While each independent heart  
muscle has an inherent faculty for independent excitation, the heartbeat is  
initiated in a specialized group of muscle cells in the sinoatrial node of the  
heart, which form the pacemaker. The heart is also innervated by nerves that  
regulate the beat. When these nerves are active, they liberate chemicals such  
25 as noradrenaline or acetylcholine from their terminals, and these  
neurotransmitters affect the cardiac muscles directly. The pacemaker is  
inhibited by acetylcholine and excited by noradrenaline.

The release of acetylcholine (ACh) opens a  $K^+$  channel in the atrium,  
slowing the rate of depolarization that leads to initiation of the action potential.

This effect is mediated through a G-protein signal transduction pathway, involving a pertussis toxin-sensitive, heterotrimeric G-protein,  $G_k$ , probably belonging to the  $G_i/G_o$  family. Activation of the  $K^+$  channel by  $G_k$  does not require cytoplasmic intermediates, suggesting direct coupling of one or more  
 5 G-protein subunits to the channel. However, a long-standing controversy exists as to which subunit couples to the channel. Both purified  $\beta\gamma$  subunit complex and  $\alpha$  subunits of the  $G_i/G_o$  family activate the channel in cell free, inside-out patches of atrial myocytes. Activation by the  $\alpha$  subunits occurs at lower concentrations than that by  $\beta\gamma$ , but seems to be less efficient. The relative  
 10 physiological importance of each pathway, as well as of possible involvement of the arachidonic acid pathway, is unclear.

A similar  $K^+$  channel is activated in the atrium by adenosine, ATP and epinephrine, probably also via a G-protein pathway. Furthermore, in nerve cells various 7-helix receptors, such as serotonin 5HT1A,  $\delta$ -opioid,  $GABA_B$  and  
 15 somatostatin couple to similar  $K^+$  channels, probably through direct activation by G-proteins. The similarity of the channels and signaling pathways in atrium and nerve cells is also shown by the coupling of a neuronal 5HT1A receptor (5HT1A-R) to the atrial channel, through transient expression in myocytes.

By electrophysiological and pharmacological criteria, these  $K^+$  channels  
 20 belongs to a family of inward rectifiers that conduct  $K^+$  much better in the inward than the outward direction, are blocked by extracellular  $Cs^+$  and  $Ba^{2+}$ , and are believed to possess a single-file pore with several permeant and blocking ion binding sites. Recently, the primary structures of two mammalian inward rectifier channels have been elucidated by cDNA cloning: an ATP-  
 25 regulated  $K^+$  channel from kidney, and an inward rectifier from a macrophage cell line. Both appear to belong to a new superfamily of  $K^+$  channels, with only two transmembrane domains per subunit and a pore region homologous to that of  $K^+$ ,  $Ca^{2+}$  and  $Na^+$  voltage-dependent channels.

G-protein regulated K<sup>+</sup> channels are important for the regulation of heart and nerve function. Determination of their molecular structure and regulation is therefore of great interest. Cloning of the channel protein genes and expression in a heterologous system would allow a molecular approach to investigation and manipulation of these regulatory pathways.

#### Relevant Literature

The activation of atrial K<sup>+</sup> channels by G-proteins is reviewed in Kurachi *et al.* (1992) Prog. Neurobiol. **39**:229-246; and Brown and Birnbaumer (1990) A. Rev. Physiol. **52**:197-213. Logothetis *et al.* (1987) Nature **325**:321-326 and Kurachi *et al.* (1989) Pflugers Arch. **413**:325-327 provide evidence for activation by the  $\beta\gamma$  subunits. Codina *et al.* (1987) Science **236**:442-445 show activation by the  $\alpha$ -subunit. Karschin *et al.* (1991) P.N.A.S. **88**:5694-5698 demonstrates the coupling of a neuronal receptor to the atrial K<sup>+</sup> channel.

Physiological characterization of the atrial K<sup>+</sup> channels is reviewed by Hille, B. (1992) *Ionic Channels of Excitable Membranes*, 2nd edition (Sinauer, Sunderland, Mass). The role of Mg<sup>2+</sup> in blocking K<sup>+</sup> efflux is discussed in Horie and Irisawa (1987) Am. J. Physiol. **253**:H210-H214.

The sequence characterization of a mammalian inward rectifier K<sup>+</sup> channel is disclosed in Ho *et al.* (1993) Nature **362**:31-38; and Kubo *et al.* (1993) Nature **362**:127-132. A brief review of these inward rectifying K<sup>+</sup> channels may be found in Aldrich (1993) Nature **362**:107-108.

#### SUMMARY OF THE INVENTION

Compositions and methods are provided for producing functional mammalian inward rectifier, G-protein activated potassium channels (Kir3.0 channels). The functional channels comprise one or more polypeptides from the Kir3.0 subfamily of inward rectifier potassium channels. The effect that

G-protein subunits exert on channel activity is determined by the specific composition of polypeptides in the channel. The functional Kir3.0 channels are useful in drug screening assays directed to modulation of cellular electrophysiology. Nucleic acids encoding Kir3.0 polypeptides are useful for expression of the gene product, and for identification of homologous genes from other species, as well as other members of the same family of proteins.

#### BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 shows inward currents evoked by high  $K^+$ , 5HT and ACh in RNA-injected oocytes. (A)  $I_{hK}$  and  $I_{5HT}$  in an oocyte injected with atrial RNA + 5HT1A-R RNA. Holding potential in this and all following figures was -80mV. (B) Inward currents evoked by ACh (AcCho) and 5HT in a single oocyte in hK solution. (C) The dependence of  $I_{5HT}$  amplitude on 5HT concentration in oocytes of one frog. In each oocyte, the response to one 5HT concentration was tested. Data represent mean $\pm$ SEM in 4-6 cells at each concentration.

Figure 2.  $I_{hK}$  and  $I_{5HT}$  are inwardly rectifying  $K^+$  currents. (A) Currents evoked by voltage steps from the holding potential of -80 mV to voltages between -140 and 40 mV in 20 mV steps in ND96(a), hK (b), hK in the presence of 5HT (c). Net  $I_{5HT}$  (d) was obtained by digital subtraction of (b) from (c). (B) Current-voltage relations of the total membrane current in a representative oocyte in NG 96 (2 mM  $[K_{out}]$ ;  $\square$ ), in 25 mM  $[K^+_{out}]$   $\blacklozenge$ ; in 75 mM  $[K_{out}]$   $\circ$ , and in hK (96 mM  $[K_{out}]$ ;  $\blacktriangle$ ). (C) Current-voltage relation of the net  $I_{5HT}$  in the same oocyte as in (B) in 25 mM  $[K_{out}]$   $\blacklozenge$ , 75 mM  $[K_{out}]$   $\circ$ , and 96 mM  $[K_{out}]$   $\blacktriangle$ . (D) The dependence of the reversal potentials of total membrane current  $\blacktriangle$  and of  $I_{5HT}$   $\bullet$  on  $[K_{out}]$ . The straight lines represent least square fits to data (mean $\pm$ SEM, n=3 for each point).

Figure 3.  $Ba^{2+}$  block of  $I_{hK}$  and  $I_{5HT}$ . (A-C), records taken from the same oocyte at 10 min intervals. Between the records, the cell was bathed in ND96.



5HT concentration was 4 nM. Note that in (B) 300  $\mu$ M Ba<sup>2+</sup> reduces I<sub>hK</sub> and almost completely blocks I<sub>5HT</sub>. Ba<sup>2+</sup> and 5HT were washed out simultaneously, and this resulted in an inward current "tail". (D) dose dependence of Ba<sup>2+</sup> inhibition of I<sub>hK</sub> in native oocytes ○, I<sub>hK</sub> in RNA-injected oocytes ●, I<sub>5HT</sub> in RNA-injected oocytes ▽. Data are mean±SEM, n=3 to 7 for each point.

Figure 4. I<sub>5HT</sub> is mediated by activation of a G-protein. (A) The effect of PTX treatment (500 ng/ml, 20-26 h) on I<sub>hK</sub> and I<sub>5HT</sub>. The cells were injected with 120 ng/oocyte total atrial RNA, 11 ng/oocyte 5HT1A-R RNA, and, where indicated, with 11 ng/oocyte G<sub>i2</sub>α RNA. (B) GDP-β-S injection inhibits I<sub>5HT</sub> but not I<sub>hK</sub> in an oocyte injected with atrial + 5HT1A-R RNAs. 5HT concentration was 0.4  $\mu$ M. A small outward current deflection (denoted by ★) upon washout of 5HT was caused by an inadvertent perfusion of ND96 for a few seconds.

#### 15 DATABASE REFERENCES AND NOMENCLATURE FOR SEQUENCES

Systematic nomenclature for the family of inward rectifying potassium channel proteins has been proposed by Doupnik *et al.* (1995) Curr. Opin. Neur. 5:268-277. This family is characterized by the presence of only two transmembrane domains per subunit, and a pore region homologous to that of K<sup>+</sup>, Ca<sup>2+</sup> and Na<sup>+</sup> voltage-dependent channels. As suggested by Doupnik *et al.*, the G-protein regulated members of this family are grouped into the Kir3 subfamily, where "Kir3.0" designates the subfamily of genes and polypeptides, and Kir3.1, 3.2, *etc.* refer to the specific subfamily members. Other subfamilies include the ATP regulated channels predominantly expressed in the kidney (Kir1.0 subfamily), and the constitutively active, steep inward rectifying Kir2.0 subfamily.

“GIRK1/KGA” and “KGB” are renamed as Kir3.1. The Genbank accession numbers for these sequences, from different species, are L25264; U01071; U01141; and D45022.

5 “GIRK2” is renamed as Kir3.2. The Genbank accession numbers for these sequences, from different species, are U011860 and U24660. “GIRK3” is renamed as Kir3.3. The Genbank accession number for this sequence is U11860. “GIRK4”, “rcKATP/CIR”, “hcKATP” is renamed Kir3.4. The Genbank accession numbers for these sequences, from different species, are X83584; L35771; X83582; and L47208.

10 A large number of heterotrimeric G-proteins have been characterized. A review of the family and database accession numbers may be found in Watson and Arkinstall (1994) The G-Protein Linked Receptor FactsBook, Academic Press, Inc., San Diego, CA, pp.296-355.

## 15 DETAILED DESCRIPTION OF THE INVENTION

Functional mammalian inward rectifier, G-protein activated potassium channels (Kir3.0 channels) are produced by expression of Kir3.0 genes. The multimeric channels vary in their response to G-protein activation, depending on the specific combination of Kir3.0 polypeptides. Drug screening assays  
20 directed to modulation of cellular electrophysiology are performed with isolated channels, membrane patches comprising channels, intact cells expressing heterologous channels, etc. Nucleic acids encoding Kir3.0 polypeptides are useful for expression of the gene product, and for identification of homologous genes from other species, as well as other members of the same family of  
25 proteins.

As used herein, the term “Kir3.0 channel” designates a functional multimeric protein that comprises one or more Kir3.0 polypeptides, preferably at least two different Kir3.0 polypeptides, e.g. Kir3.1, Kir3.2, Kir3.3, etc., where

the polypeptides may be from the same or different species. The functional channel has the distinctive features of an inward rectifier, in that it conducts inward but not outward  $K^+$  current; it is blocked by low concentrations of extracellular  $Cs^+$  or  $Ba^{2+}$ ; and the conductance of the channel does not depend solely on voltage, but on  $(E-E_K)$ . Characteristic of the Kir3.0 family, the ability of the channel to conduct  $K^+$  current is modulated by G-proteins, particularly G-proteins of the  $G_i/G_o$  family. It has been found that such modulation may be mediated directly by the G-protein  $\beta\gamma$  subunit. It will be understood by one of skill in the art that a number of mammalian cell surface receptors activate G-proteins as a consequence of specific ligand binding. The signal transduction from receptor to Kir3.0 channel can therefore be coupled through G-protein intermediates.

The specific combination of Kir3.0 polypeptides in the channel will determine the properties of the channel. As an example, it has been observed that a channel comprised of Kir3.1 and Kir3.2 polypeptides, or of Kir3.1 and Kir3.3 polypeptides, demonstrates a significant enhancement of the G-protein evoked currents. In contrast, a channel comprised of Kir3.2 and Kir3.3 polypeptides shows a decrease in G-protein evoked currents.

As used herein, the term Kir3.0 polypeptide is used to designate a single polypeptide that is capable of associating with other Kir3.0 polypeptides to form a functional Kir3.0 channel as defined above. When a nucleic acid encoding a Kir3.0 polypeptide is introduced into a cell, particularly a *Xenopus* oocyte, and expressed, it confers a change in the electrophysiology of the cell, such that the cell has an altered ability to conduct  $K^+$  current, particularly in response to G-protein activation. Genes encoding Kir3.0 polypeptides can be identified in such a system based on the expression of a single gene, or a combination of genes. The ability of Kir3.0 polypeptides to associate and form multimeric channels in a cell provides a means of identifying genes encoding

novel Kir3.0 polypeptides, where a candidate gene may be introduced into a cell comprising a known Kir3.0 gene. A change in the electrophysiology of the cell, as described above, is indicative that the candidate gene encodes a Kir3.0 polypeptide able to partake in multimeric channel formation.

- 5 Kir3.0 polypeptides may also be characterized by sequence similarity to known members of the Kir3.0 family. SEQ ID NO:2 describes an exemplary Kir3.1 polypeptide, of rat origin. Within a species, various members of the Kir3.0 gene family will usually have at least about 50% amino acid sequence identity, more usually at least about 60%, and may be as high as 70 to 80%.
- 10 Between mammalian species, the homologous Kir3.0 polypeptides, e.g. human and rat Kir3.1, etc., have a high degree of similarity, usually at least about 75% amino acid sequence identity, more usually at least about 85% sequence identity, and may be as high as 90% sequence identity, particularly in the conserved transmembrane and pore forming regions.
- 15 For convenience in experimental manipulation and drug screening assays, the Kir3.0 channel may be assembled in an expression host cell. As used herein, an expression host cell is a cell, preferably a eukaryotic cell having substantially no endogenous Kir3.0 channels. The expression host will have introduced into it one or more different exogenous nucleic acid(s)
- 20 encoding one or more Kir3.0 polypeptides, preferable at least two polypeptides. Such exogenous nucleic acids will be capable of constitutive or inducible expression of the Kir3.0 nucleic acid(s) in the host cell. Where two or more polypeptides are present, the genes encoding Kir3.0 polypeptides may be on a single vector or DNA molecule, or may be present on separate molecules. The
- 25 expression of the exogenous nucleic acid causes an altered ability to conduct  $K^+$  current in the expression host cell, particularly in response to G-protein activation. For example, nucleic acids encoding one or more mammalian Kir3.0 polypeptides can be inserted into a host cell, e.g. *Xenopus* oocyte; yeast;

mammalian immortalized cell line or primary cell culture; plant cell; *etc.* by transfection, injection, transduction, *etc.* Expression of the nucleic acid produces Kir3.0 polypeptides, which will assemble into a channel in the membrane of the host cell. It has been found in some cases that the heterologous Kir3.0 polypeptide will assemble with polypeptides of the heterologous host cell to form a hybrid channel. Cell surface receptors, such as muscarinic receptors, serotonin receptors, *etc.*, and G-proteins may additionally be introduced into the heterologous host cell. A functional heterologous channel will regulate inward  $K^+$  current in response to the presence of exogenous or endogenous activated G-proteins.

The Kir3.0 channels in homologous or heterologous cells are useful in the study of cell electrophysiology, including but not limited to neural and cardiac cells, particularly G protein linked responses, such as those coupled to acetylcholine and serotonin receptor binding. Of particular interest are *in vitro* drug screening assays. Screening assays are provided to identify drug candidates that specifically alter Kir3.0 channel activity either directly or indirectly by modulating receptors, G-proteins, channels or the interactions among these elements. Novel binding agents include specific antibodies, natural or non-natural binding agents identified in screens of chemical libraries, analogs of acetylcholine, G proteins, *etc.* Areas of investigation include the development of cardiovascular, neurologic endocrine and gastrointestinal treatments.

Nucleic acid molecules are provided that encode individual polypeptides capable of forming a Kir3.0 channel. The subject polypeptide may be any one of Kir3.1 (for purposes of continuity referred to hereafter as Kir3.1/KGA), Kir3.2, Kir3.3, Kir3.4, *etc.* The origin of the nucleic acid may be any mammalian species, *e.g.* human, murine, primate, bovine, equine, canine, feline, ovine, porcine, *etc.*



The subject nucleic acids may be used in a variety of ways. They may be used as probes for identifying other Kir3.0 genes. Homologous sequences and related members of the Kir3.0 gene family are those with substantial sequence similarity to the subject sequences, as previously described.

5 Algorithms for sequence analysis are known in the art, and include BLAST, described in Altschul *et al.* (1990) J Mol Biol **215**:403-10; ADVANCE and ADAM, described in Torelli and Robotti (1994) Comput Appl Biosci **10**:3-5; and FASTA, described in Pearson and Lipman (1988) P.N.A.S. **85**:2444-8.

Such homologous or related nucleic acid sequences are detected by  
10 hybridization under low stringency conditions, for example, at 50° C and 10XSSC (0.9 M saline/0.09 M sodium citrate) and remain bound when subject to washing at 55°C with 1XSSC. The high degree of similarity between Kir3.0 homologs allows the use of relatively stringent hybridization conditions, as known in the art. For example, see Sambrook *et al. Molecular Cloning* (Cold  
15 Spring Harbor, New York, 1989). By using probes, particularly labeled probes of DNA sequences, one may be able to isolate homologous genes, which may be then used for identifying members of the Kir3.0 family in other species. By probes is intended a single stranded oligonucleotide of at least about 12 nt in length, more usually at least about 15 nt, and preferably at least about 18 nt,  
20 characterized by the ability to hybridize under low stringency conditions to Kir3.0 genes. The term "unique" as used herein defines a nucleic acid molecule that does not contain known genomic repeated sequences, including but not limited to *Alu* sequences.

Various methods are known in the art for identification of nucleic acids  
25 having sequence similarity, based on the ability of single stranded nucleic acids, e.g. DNA to form a double stranded complex. A sample may be screened for the presence of nucleic acids capable of forming double stranded complexes with a Kir3.0 probe, particularly under low stringency conditions.

5 "Sample" as used herein includes but is not limited to genomic libraries, cDNA libraries, nucleic acid molecule extracts from tissue, or nucleic acid molecule extracts from cell culture. After complex formation, the other nucleic acid molecule is isolated by methods known in the art. Methods include the polymerase chain reaction (PCR) or reverse transcriptase PCR (RT-PCR), where two probes from the subject nucleic acids sequence are used to amplify a region of the sample DNA or cDNA. PCR may also be performed with a single Kir3.0 probe, and a second oligonucleotide complementary to a known genomic repeat sequence. Alternatively, northern or southern blots are useful in identifying bands, colonies, plaques, *etc.* comprising nucleic acids capable of hybridizing to a Kir3.0 probe. Generally the Kir3.0 probe will be labeled with a detectable marker, *e.g.*  $^{32}\text{P}$ ,  $^{35}\text{S}$ , biotin, FITC, *etc.* when used in a northern or southern blot.

15 The subject nucleic acids may also be used to identify the expression of Kir3.0 genes in a biological specimen. The manner in which one probes cells for the presence of particular nucleotide sequences, particularly as genomic DNA, is well-established in the literature and does not require elaboration here. Conveniently, a biological specimen is used as a source of mRNA. The mRNA may be amplified by polymerase chain reaction using primers specific for the subject DNA sequences. Alternatively, the mRNA sample is separated on gel electrophoresis and then probed using Northern blotting with a fragment of the subject DNA as a probe. Other techniques may also find use. Detection of mRNA having the subject sequence is indicative of Kir3.0 expression in the sample.

25 The subject nucleic acid sequences may be modified for a number of purposes, particularly where they will be used intracellularly, for example, by being joined to a nucleic acid cleaving agent, *e.g.* a chelated metal ion, such as iron or chromium for cleavage of the gene; as an antisense sequence; or the



like. Modifications may include replacing oxygen of the phosphate esters with sulfur or nitrogen, replacing the phosphate with carboxamide, phosphoramidate, etc.

5 The DNA sequence may encode amino acid sequences that differ from the native sequence of a Kir3.0 polypeptide, but that do not produce functional changes in the Kir3.0 polypeptide. The sequence may encode polypeptide analogs, fragments or derivatives of substantially similar polypeptides that differ from the naturally-occurring forms in terms of the identity of location of one or more amino acid residues (deletion analogs containing less than all of the  
10 residues specified for the protein, substitution analogs wherein one or more residues are replaced by other residues and addition analog wherein one or more amino acid residues is added to a terminal or medial portion of the polypeptides) and which share some or all properties of naturally-occurring forms. These sequences include the incorporation of preferred codons for expression in non-mammalian host cells; the provision of sites for cleavage by  
15 restriction endonuclease enzymes; the addition of promoters operatively linked to enhance RNA transcription; and the provision of additional initial, terminal or intermediate DNA sequences that facilitate construction of readily expressed vectors.

20 For expression, the DNA sequences may be inserted into an appropriate expression vector, where the native transcriptional initiation region may be employed or an exogenous transcriptional initiation region, *i.e.* a promoter other than the promoter which is associated with the gene in the normally occurring chromosome. The promoter may be introduced by recombinant  
25 methods *in vitro*, or as the result of homologous integration of the sequence into a chromosome. A wide variety of transcriptional initiation regions are known for a wide variety of expression hosts, where the expression hosts may involve prokaryotes or eukaryotes, particularly *E. coli*; *B. subtilis*; yeast cells;

mammalian cells; e.g. Cos cells, HeLa cells, L(tk-), primary cultures; insect cells; *Xenopus laevis* oocytes; and the like. Generally a selectable marker operative in the expression host will be present. The promoter may be operably linked to the coding sequence of the genes of interest so as to produce a translatable mRNA transcript. Expression vectors have convenient restriction sites located near the promoter sequence so as to provide for the insertion of nucleic acid sequences encoding heterologous proteins. The promoters in suitable expression vectors may be either constitutive or inducible. Expression vectors for the production of fusion proteins, where the exogenous fusion peptide provides additional functionality, *i.e.* increased protein synthesis, stability, reactivity with defined antisera, an enzyme marker, e.g.  $\beta$ -galactosidase, *etc.*

Expression vectors may also be used to produce mRNA encoding a Kir3.0 polypeptide. The mRNA may be isolated, and used for synthesis of the polypeptide by injecting the RNA molecules into *Xenopus* oocytes and culturing the oocytes under conditions that are well known to an ordinary artisan.

The subject nucleic acids are used to identify nucleic acids encoding receptors and G-proteins that modulate the activity of Kir3.0 channels by activating or deactivating the channel. As previously described, such methods may also be used to identify genes encoding novel Kir3.0 polypeptides as well. A candidate nucleic acid or library of nucleic acids may be introduced into an expression host cell comprising a Kir3.0 channel, and the host cell screened for a change in the Kir3.0 channel activity. The candidate nucleic acid or library of nucleic acids may be selected from a variety of sources, including cDNA expression constructs operable in the host cell or mRNA transcribed *in vivo* or *in vitro* and subsequently introduced into the host cells. Pools of different nucleic acids may be tested, where positive pools are then used to generate smaller pools or individual clones. Nucleic acids that give a positive signal for

modulation of Kir3.0 channel activity are then isolated and characterized as to the nucleic acid sequence and structure. The change in channel activity may be the result of expression of the candidate nucleic acid directly, or a known activating agent may be added to the cells to effect a change in channel activity.

5           A candidate nucleic acid encoding a putative receptor or G protein that interacts with the subject channels may be tested for its ability to modulate Kir3.0 channel activity. The candidate nucleic acid is introduced into an expression host in conjunction with a Kir3.0 expression construct of one or more Kir3.0 polypeptides, preferably at least two polypeptides. The expression  
10   host is contacted with a known G-protein associated receptor activator, where an increase in Kir3.0 channel conductance indicates activation of the channel, and a decrease in conductance indicated inhibition of the channel.

          Expression cassettes may be prepared comprising a transcription initiation region, which may be constitutive or inducible, the gene encoding the  
15   subject methyltransferase or fragment thereof, and a transcriptional termination region. Of particular interest is the use of sequences which allow for the expression of functional epitopes or domains, usually at least about 24 nucleotides in length, more usually at least about 48 nucleotides in length, and up to the complete open reading frame of the gene. After introduction of the  
20   DNA, the cells containing the construct may be selected by means of a selectable marker, the cells expanded and then used for expression.

          The expression cassettes may be introduced into a variety of vectors, where the vectors will normally be characterized by the ability to provide selection of cells comprising the expression vectors. The vectors may provide  
25   for extrachromosomal maintenance, particularly as plasmids or viruses, or for integration into the host chromosome. Where extrachromosomal maintenance is desired, an origin sequence will be provided for the replication of the plasmid, which may be a low- or high-copy plasmid. A wide variety of markers

are available for selection, particularly those which protect against toxins, more particularly against antibiotics. The particular marker that is chosen will be selected in accordance with the nature of the host, where in some cases, complementation may be employed with auxotrophic hosts, e.g. bacteria, yeast.

5 Introduction of the DNA construct may be by any convenient means, e.g. conjugation, calcium-precipitated DNA, electroporation, fusion, transfection, infection with viral vectors, etc.

With the availability of the protein in large amounts by employing an expression host, the protein may be isolated and purified in accordance with conventional ways. A lysate may be prepared of the expression host and the lysate purified using HPLC, exclusion chromatography, gel electrophoresis, affinity chromatography, or other purification technique. The purified protein will generally be at least about 80% pure, preferably at least about 90% pure, and may be up to 100% pure. By pure is intended free of other proteins, as well as cellular debris.

10  
15

The polypeptide may be used for the production of antibodies. Antibodies may be prepared in accordance with conventional ways, where the expressed polypeptide or protein is used as an immunogen, by itself or conjugated to known immunogenic carriers, e.g. KLH, BSA, etc. Various adjuvants may be employed, as appropriate. For monoclonal antibodies, after one or more booster injections, the spleen of the immunized animal is isolated, the splenocytes immortalized, and then screened for high affinity antibody binding. The immortalized cells, i.e. hybridomas, producing the desired antibodies may then be expanded. For further description, see

20  
25 Monoclonal Antibodies: A Laboratory Manual, Harlow and Lane eds., Cold Spring Harbor Laboratories, Cold Spring Harbor, New York, 1988. The antibodies may find use in diagnostic assays for detection of the presence of Kir3.0 channels in patient samples.

By providing for the production of large amounts of Kir3.0 polypeptides and channels, one can identify ligands or substrates which bind to, or modulate the action of Kir3.0 channels. The subject Kir3.0 channels, polypeptides or functional domains thereof are used to screen for agonists or antagonists that modulate the K<sup>+</sup> channel activity, by increasing or decreasing the evoked K<sup>+</sup> current. In this way, drugs may be identified that can alter the electrophysiology of cells comprising Kir3.0 channels, including atrial, neural, vascular smooth muscle, endothelial, pancreatic and other cells. Of particular interest are screening assays for agents that have a low toxicity for human cells. A wide variety of assays may be used for this purpose, including whole cell or single channel current quantitation in response to stimulus, patch clamping, labeled *in vitro* protein-protein binding assays, electrophoretic mobility shift assays, immunoassays for protein binding, and the like.

In a preferred embodiment, a whole cell or single channel current will be quantitated in the presence of a candidate pharmacological agent. The assay will include contacting a cell into which has been introduced a functional Kir3.0 channel with a candidate pharmacological agent, and detecting any change in channel conductance. An increase in inward  $K^+$  channel conductance indicates channel activation, while a decrease indicates that the agent is a channel inhibitor.

The term "agent" as used herein describes any molecule, protein, or pharmaceutical with the capability of directly or indirectly altering ion channel conductance by affecting second messenger systems, receptors, G-proteins, interactions among the elements, or the ion channel directly. Agents include but are not limited to serotonin, neurotrophin, enkephalins, dopamine, arachidonic acid, cholera toxin, and pertussis toxin. Generally a plurality of assay mixtures are run in parallel with different agent concentrations to obtain a differential response to the various concentrations. Typically, one of these

concentrations serves as a negative control, *i.e.* at zero concentration or below the level of detection.

5 The term "activators" as used herein defines any agent which activates a Kir3.0 channel, G-protein associated receptor or G-protein. The term "activates" as used herein is applied to both G-protein associated receptors and ion channel conductance and in terms of G-protein associated receptors defines the state of the receptor wherein it initiates release of a G-protein subunit which in turn initiates a cellular response. In terms of the ion channel conductance "activates" defines the state of the channel wherein the channel  
10 increases conductance. The term "deactivates" as used herein defines the state of the channel wherein the channel is initiated to decrease conductance or is incapable of conductance under conditions when the channel normally conducts ions across a membrane. The term "agonist" as used herein defines an agent that initiates activation of ion channel conductance or initiates  
15 activation of a second messenger system. The term "antagonist" as used herein defines an agent that initiates deactivation of ion channel conductance or initiates deactivation of a second messenger system.

Candidate agents encompass numerous chemical classes, though typically they are organic molecules, preferably small organic compounds  
20 having a molecular weight of more than 50 and less than about 2,500 daltons. Candidate agents comprise functional groups necessary for structural interaction with proteins, particularly hydrogen bonding, and typically include at least an amine, carbonyl, hydroxyl or carboxyl group, preferably at least two of the functional chemical groups. The candidate agents often comprise cyclical  
25 carbon, heterocyclic or carbocyclic structures and/or aromatic or polyaromatic structures substituted with one or more of the above functional groups. Candidate agents are also found among biomolecules including peptides,

saccharides, fatty acids, steroids, purines, pyrimidines, derivatives, structural analogs or combinations thereof.

Candidate agents are obtained from a wide variety of sources including libraries of synthetic or natural compounds. For example, numerous means  
5 are available for random and directed synthesis of a wide variety of organic compounds and biomolecules, including expression of randomized oligonucleotides. Alternatively, libraries of natural compounds in the form of bacterial, fungal, plant and animal extracts are available or readily produced. Additionally, natural or synthetically produced libraries and compounds are  
10 readily modified through conventional chemical, physical and biochemical means. Known pharmacological agents may be subjected to directed or random chemical modifications, such as acylation, alkylation, esterification, amidification to produce structural analogs.

Various techniques for performing screening assays are known in the  
15 art. For example, a screening assay may utilize one or more molecules conjugated to a label, where the label can directly or indirectly provide a detectable signal. Various labels include radioisotopes, fluorescers, chemiluminescers, enzymes, specific binding molecules, particles, e.g. magnetic particles, and the like. A mixture of the components are added to the  
20 assay sample, and the binding of candidate agents to the subject channels, receptors or G proteins is detected. Alternatively, a host cell expressing a Kir3.0 channel is combined with a candidate agent under conditions that induce a change in K<sup>+</sup> current.

When an agent is identified that has a desired pharmacological activity, it  
25 may be used for prophylactic, therapeutic or experimental purposes. Such agents find use in investigating the interactions of inward K<sup>+</sup> channels with surface membrane receptors, G-proteins and second messenger systems. For therapeutic use, the agent may be administered in a physiologically

acceptable carrier to a host. The agents may be administered in a variety of ways, orally, parenterally *e.g.* subcutaneously, intraperitoneally, intravascularly, intralingually, topically, *etc.* Depending upon the manner of introduction, the compounds may be formulated in a variety of ways, where the concentration of therapeutically active compound in the formulation may vary from about 0.1-100 wt.%. 5

For convenience, a kit may be provided having the components necessary to perform the subject screening assays. Such a kit may contain at least one expression vector comprising a Kir3.0 gene. Usually at least two 10 Kir3.0 genes will be included, which may be on one vector or two vectors. The kit may further comprise agents for the detection of K<sup>+</sup> current change, and agents having a known positive or negative effect on activity of Kir3.0 channels or receptors associated therewith.

15 The following examples are offered by way of illustration and not by way of limitation.

## EXPERIMENTAL

### Isolation of a cDNA encoding Kir3.1/KGA

20 A cDNA plasmid library was made from 19-day-old rat atrial mRNA. The library was linearized and amplified by polymerase chain reaction using primers complementary to sequences flanking the cDNA insert. cRNA was synthesized *in vitro* from the T7 promoter and microinjected into *Xenopus laevis* oocytes. Electrophysiological recordings identified an inward rectifier, 25 G-protein activated, potassium channel.

### Materials and Methods

Preparation of RNA and oocytes. Total RNA was extracted from atria and ventricles of 19-21 day old rats of both sexes using the procedure of



Chomczynski and Sacchi (1987) Anal. Biochem. **162**:156-159. Poly (A) RNA was separated on an oligo-dT cellulose column (type 3, Collaborative Biochemical Products). Ventricle poly(A) RNA was fractionated by centrifugation (18 h, 30,000 g, 4°C) on a linear 5%-25% sucrose gradient.

5 *Xenopus laevis* oocytes were prepared as previously described (Dascal & Lotan (1992) in *Methods in Molecular Biology*, v. 13: *Protocols in Molecular Neurobiology*, eds. Longstaff & Revest). and injected with either 50-120 ng/oocyte poly(A) RNA, 120-200 ng/oocyte total RNA, or 35 ng/oocyte fractionated poly(A) RNA. In most cases, 5HT1A-R RNA (5-20 ng/oocyte) was  
10 co-injected with atrial or ventricle RNA. Final volume of the injected RNA solution was 50 nl. The oocytes were incubated for 3-7 days in the NDE solution (ND96 (see below) containing 1.8 Mm CaCl<sub>2</sub> and supplemented with 2.5 Mm Na-pyruvate and 50 µg/ml gentamicin). Occasionally, either 2.5-5% heat-inactivated horse serum or 0.5 mM theophylline were added to the NDE  
15 solution. Incubation of oocytes in pertussis toxin (PTX; List Biochemicals) was done in NDE solution without the addition of pyruvate, serum or theophylline. cDNAs of 5HT1A receptor (Karschin *et al.* (1991) P.N.A.S. **88**:5694-5698) and G<sub>i2a</sub> (a gift from M. I. Simon, Caltech) in pBluescript were linearized, and RNA was synthesized *in vitro* as described (Dascal and Lotan, *supra.*).

20 Electrophysiological recordings were performed using the two electrode voltage clamp method with the Dagan 8500 amplifier (Dagan Instruments, Minneapolis) as described (Dascal *et al.* (1986) Mol. Brain Res. **1**:201-209). The oocytes were usually kept in the ND96 solution: 96 mM NaCl/2 mM KC1/1 mM MgCl<sub>2</sub>/1 mM CaCl<sub>2</sub>/5 mM Hepes, pH=7.5. Most measurements were done  
25 in the high K<sup>+</sup> solution (hK): 96 mM KCl/2 mM NaCl/1 mM MgCl<sub>2</sub>/1 mM CaCl<sub>2</sub>/5 mM Hepes, pH=7.5. Solutions containing intermediate concentrations of K<sup>+</sup> were made by substituting K<sup>+</sup> for Na<sup>+</sup>. Solution exchange and drug application were done by superfusing the cell placed in a 0.5 ml chamber. GDP-β-S

(trilithium salt; Sigma) was injected by pressure (Dascal *et al. supra.*). Stimulation, data acquisition, and analysis were performed using pCLAMP software (Axon Instruments, Foster City, CA).

5

## RESULTS

To express the KG channel, the oocytes were injected with atrial total or poly(A) RNA. In order to avoid the possibility that a low level of expression of the muscarinic receptor will make undetectable even a well-expressed KG channel, atrial RNA was usually supplemented with mRNA coding for the serotonin-5HT1A receptor (5HT1A-R); oocytes injected with this RNA mixture will be termed RNA-injected oocytes throughout the paper. When expressed in atrial myocytes, the 5HT1A-R efficiently coupled to the KG channel normally existing in these cells (Karschin *et al. supra.*), and it was expected to do so in the oocytes.

Four to 5 days after RNA injection addition of 10  $\mu$ M ACh or 1-2 $\mu$ M 5HT to the ND96 bath solution did not cause any significant change in membrane current. Therefore, the effects of ACh and 5HT were tested in a high potassium (hK) solution with 96 mM K<sup>+</sup> and 2 mM Na<sup>+</sup>. In this solution, the K<sup>+</sup> equilibrium potential ( $E_K$ ) is close to 0 mV, and this enables inward K<sup>+</sup> current flow through inwardly rectifying K channels at negative holding potentials (-80 mV was routinely used in this study).

Changing ND 96 to the hK solution was accompanied by the development of an inward current that reached a steady level within 0.5-1 min ( $I_{hK}$ ; Fig 1A).  $I_{hK}$  was also observed in native (not injected with any RNA) oocytes, or in oocytes injected with 5HT1A-R RNA alone, but it was always larger in RNA-injected oocytes ( $P < 0.001$ , two-tailed t-test; Table 1).

Table 1

Inward currents evoked by high  $K^+$  and by 5HT. The entries are inward currents in nA shown as mean $\pm$ SEM (n), measured at -80mV in the hK solution. 5HT concentration ranged in different experiments from 100 nM to 2  $\mu$ M.

Injected RNA	$I_{hK}$	$I_{5HT}$
None (native oocytes)	72 $\pm$ 6 (34)	0 (18)
5HT1A-R	54 $\pm$ 4 (11)	0 (12)
Atrial + 5HT1A-R	123 $\pm$ 8 (55)	290 $\pm$ 43 (55)

5 In RNA-injected oocytes, application of 5HT or ACh in hK solution induced an inward current ( $I_{5HT}$ ) that subsided upon washout of the transmitter (Fig. 1A, B). The response to ACh was usually smaller than to 5HT when measured in the oocytes of the same frog (Fig. 1B). Thus, in oocytes of one frog  $I_{5HT}$  was 1102 $\pm$ 84 nA (n=6), whereas the ACh response was 382 $\pm$ 45  
10 nA(n=6).  $I_{5HT}$  tended to decrease on repeated applications of 5HT, and this could be overcome by increasing the intervals between applications to 10 min or more, suggesting the presence of a desensitization process.  $I_{5HT}$  and an increased (in comparison with native oocytes)  $I_{hK}$  were also observed in oocytes injected with ventricle poly (A) RNA + 5HT1A-R RNA, but the  $I_{5HT}$  was  
15 about 20 times smaller than with atrial poly(A) RNA (not shown). 5HT had no effect in oocytes injected with atrial RNA without the 5HT1A-R RNA (n=4) or with 5HT1A-R RNA alone, or in native oocytes (Table 1).

The 5HT dose-response curve showed saturation at about 100 nM and a half-maximal response at about 15 nM (Fig. 1C), which is characteristic of the  
20 5HT1 receptor class (Hoyer & Schoeffer (1991) J. Recept. Res. 11:197-214). A similar current was evoked by a selective 5HT1A agonist, 8-OH DPAT (8-OH-2(D1-n-(propylamino)-tetralin).

The current-voltage (I-V) characteristic of the oocyte membrane was studied by applying voltage steps from a holding potential of -80 mV. In normal

ND96, in the range -140 - -20 mV, only voltage- and time-independent "leak" currents were observed (Fig. 2a), and the I-V curve was linear (Fig. 2B). Above -20 mV, a slowly developing outward current was observed (Fig. 2A, a-c); this is known to be due to opening of a  $\text{Cl}^-$  channel activated by  $\text{Ca}^{2+}$  entry through voltage-dependent  $\text{Ca}^{2+}$  channels (Barish (1983) J. Physiol. **342**:309-325). The  $\text{Ca}^{2+}$ -activated  $\text{Cl}^-$  current was also seen in the hK solution; in addition, the total membrane current evoked by steps to -120 and up to -20 mV was larger than in ND96 (Fig. 2Ab; 2B), whereas above 0 mV there was little or no change. This suggested that most or all of  $I_{\text{hK}}$  elicited at -80 mV by the exchange of ND96 to hK solution was due to a  $\text{K}^+$  current flowing through a constitutively active inward rectifier  $\text{K}^+$  channel(s). This current showed some time-dependent inactivation at -140 mV (Fig. 2Ab) and at more negative potentials; this inactivation phenomenon was not studied further. In the presence of 5HT, the membrane currents between -140 and -20 mV were further increased (Fig. 2Ac). Net 5HT-evoked currents, obtained by digital subtraction of total membrane currents in the absence of 5HT from currents in its presence (Fig. 2Ad), showed clear inward rectification; the 5HT-activated channels conducted little or no current above  $E_{\text{K}}$  at different external  $\text{K}^+$  concentrations,  $[\text{K}_{\text{out}}]$  (Fig. 2C). The extrapolated reversal potential of  $I_{5\text{HT}}$  showed an almost perfect selectivity of the 5HT-activated channel to  $\text{K}^+$ , changing by about 58 mV per 10-fold change in  $[\text{K}_{\text{out}}]$  (Fig. 2D). The reversal potential of the total membrane current in the absence of 5HT also depended on  $[\text{K}_{\text{out}}]$  (Fig. 2B) but changed only by 24 mV per tenfold change in  $[\text{K}_{\text{out}}]$  (Fig. 2D). This does not necessarily imply poor ion selectivity of the constitutively active inward rectifier, but may reflect the relatively high contribution of  $\text{Cl}^-$  and  $\text{Na}^+$  to the resting membrane conductance (Dascal *et al.* (1984) J. Physiol. **352**:551-574).

Block by external  $Ba^{2+}$  is one of the characteristic features of inward rectifiers (Hille, *supra.*). In normal ND96 solution,  $Ba^{2+}$  (5  $\mu$ M-3 mM) did not cause any significant changes in resting current or conductance in native or RNA-injected oocytes at the holding potential of -80mV. In the hK solution,  $Ba^{2+}$  inhibited both  $I_{hK}$  and  $I_{5HT}$  (Fig. 3), and this was accompanied by a decrease in membrane conductance. 300  $\mu$ M,  $Ba^{2+}$  blocked about 20% of  $I_{hK}$  but almost completely abolished  $I_{5HT}$  (Fig. 3B). The  $IC_{50}$  (half-inhibition concentration) for  $Ba^{2+}$  block of  $I_{5HT}$  was about 15  $\mu$ M, whereas  $IC_{50}$  for  $I_{hK}$  block was above 3 mM (Fig. 3D). It is noteworthy that, although the sensitivity of  $I_{hK}$  to  $Ba^{2+}$  block was similar in native and RNA-injected oocytes, the latter did appear to have a small component of  $I_{hK}$  inhibited by low doses of  $Ba^{2+}$  (Fig. 3D). This raises the possibility that the atrial  $I_{hK}$  is more sensitive to  $Ba^{2+}$  block than the oocyte's  $I_{hK}$ , or that a fraction of the highly  $Ba^{2+}$ -sensitive channels underlying  $I_{5HT}$  could be active in the absence of agonist. Note also that there was an inward current "tail" observed when  $Ba^{2+}$  and 5HT was washed out simultaneously (Fig. 3B), presumably because the rate-limiting step in deactivation of the channel proceeds more slowly than unblock from  $Ba^{2+}$ .

To estimate the size of RNA encoding the expressed inward rectifiers, ventricle poly(A) RNA was fractionated on a sucrose gradient. The size distribution of the fractions was measured by RNA gel blots probed with [ $^{32}$ P]-labeled poly(T) (Lubbert *et al.* (1987) J. Neurosci. 7:1159-1165). The RNA encoding  $I_{5HT}$  was found mainly in two size fractions covering the range between 2.5 and 5.5 kb. The peak expression of ventricle  $I_{hK}$  was in lower size fractions, in the 1.5-3 kb range.

In atrium, the muscarinic receptor is coupled to the KG channel via a PTX-sensitive G-protein. Surprisingly, in RNA-injected oocytes,  $I_{5HT}$  was not affected by treatment with PTX; neither was  $I_{hK}$  (Fig. 4A). To test whether the

5HT1A receptor couples to the K<sup>+</sup> channel via a G-protein, the oocytes were injected with 400-800 pmole/oocyte of the non-hydrolysable analog of GDP, GDP-β-S, which is known to inhibit the activity of PTX-sensitive as well as of PTX-insensitive G-proteins (Gilman (1987) A. Rev. Biochem. 56:615-649). In 4 cells, GDP-β-S injection had no effect on I<sub>hK</sub> (115±8% of control) but strongly inhibited I<sub>5HT</sub>, to 4±1% of control (Fig. 4B). Thus, it appears that the coupling between the 5HT1A receptor and the KG channel occurs via an oocyte's endogenous PTX-insensitive G-protein.

We examined whether an overexpressed PTX-sensitive α subunit of a G-protein, e.g. G<sub>i2</sub>α, could compete with the "native" PTX-insensitive α subunit for the expressed 5HT1A receptor, thus restoring the PTX sensitivity of the KG channel activation. As shown in Fig. 4A, in oocytes injected with atrial RNA plus cRNAs encoding 5HT1A-R and G<sub>i2</sub>α, PTX inhibited I<sub>5HT</sub> by about 50% (P<0.01, two-tailed t-test), whereas I<sub>hK</sub> was unaffected.

## DISCUSSION

The present results demonstrate for the first time that the atrial inward rectifier K<sup>+</sup> (KG) channel, which in the native tissue is activated by ACh via a PTX-sensitive G-protein, is expressed in oocytes injected with atrial RNA. Current through the channel can be activated by acetylcholine (ACh) or, if RNA encoding a neuronal 5HT1A receptor is co-injected with atrial RNA, by serotonin (5HT). Activation of the channel probably occurs via a muscarinic ACh receptor synthesized following atrial RNA injection, rather than via the oocyte's endogenous muscarinic receptor. The latter couples to phospholipase C, and its activation induces very characteristic large transient Cl<sup>-</sup> current responses caused by Ca<sup>2+</sup> release from intracellular stores (Dascal (1987) CRC Crit. Rev. Biochem. 22:317-387). Fortunately, the majority of oocyte batches lose this response after defolliculation (Miledi & Woodward

00039927-034600  
(1989) J. Physiol. **416**:601-621), and this response was not observed in the present study. Because the ACh-evoked currents were small in most cases, we concentrated on the study of the 5HT response; the latter was undoubtedly mediated by the introduced 5HT1A receptor, as 5HT was ineffective in oocytes not injected with 5HT1A-RNA, and the response displayed the expected pharmacological properties.

The evidence presented here indicates that, in oocytes injected with atrial and 5HT1A-R RNAs, activation of the 5HT1A receptor leads to opening of a K<sup>+</sup> channel that bears distinctive features of an inward rectifier, similar to those of the atrial KG: i) it conducts inward but not outward K<sup>+</sup> current; ii) it is blocked by low concentrations of Ba<sup>2+</sup>, iii) the conductance of the channel does not depend solely on voltage but on (E-E<sub>K</sub>). The expression of this channel must truly be directed by atrial RNA, because: i) no hormone or transmitter-activated current of this kind is observed in native oocytes; ii) expression of 5HT1A receptor alone does not cause the appearance of such a response. Based on ventricle RNA fractionation data, the RNA encoding the 5HT-activated channel is in a broad size range between 2.5 and 5.5 kb. This is similar or somewhat smaller than the reported 4-5 kb mRNA size of some constitutively active inward rectifiers expressed in *Xenopus* oocytes (Lewis *et al.* (1991) FEBS Lett. **290**:17-21; and Perier *et al.* (1992) J. Neurochem. **59**:1971-1974), as well as of the cloned IRK1 (5.5 kb) and ROMK1 (4 kb) channels. The properties of I<sub>5HT</sub> directed by ventricle and atrial RNA are very similar, and it is reasonable to assume that they are encoded by the same RNA species.

Opening of the inward rectifier by 5HT is mediated by activation of a G-protein, as expected for the KG channel, because i) 5HT1A receptor belongs to the family of 7-helix receptors all of which act via G-proteins (Dascal (1987) *supra.*); ii) I<sub>5HT</sub> was inhibited by intracellular injection of GDP-β-S. However, the G-protein participating in this pathway was PTX-insensitive, possibly an

endogenous oocyte G-protein. It is not clear why in the oocyte the channel activation pathway involves a PTX-insensitive G-protein. The atrial KG channel normally couples to  $G_i$  (Brown & Birnbaumer, *supra.*), and there are at least two subspecies of  $G_i$  in the oocyte (Olate *et al.* (1990) FEBS Lett. **268**:27-31); also, some  $G_i$  may be expressed from atrial RNA. Also, in the hippocampus, the 5HT<sub>1A</sub> receptor opens a  $K^+$  channel by activating a PTX-sensitive G-protein. One possibility is that a vast excess of this undefined PTX-insensitive G-protein overrides the others in competition for coupling to the 5HT<sub>1A</sub> receptor. Whatever the reason for this unexpected coupling, our results show that the PTX sensitivity of the KG channel activation can be partially restored by overexpression of the  $\alpha$  subunit of  $G_i$ . Since the actual identify of the  $\alpha$  subunit does not seem to be important for activation of the expressed KG channel, these results imply that the  $\beta\gamma$  subunit complex doublet may be the activator of the channel in this case.

Atrial and ventricle RNAs also induce an enhanced activity of an additional inward rectifier, that is active in the absence of any specific stimulation (referred to as  $I_{hK}$  herein).  $I_{hK}$  in atrial RNA-injected oocytes is about twice as large as in native oocytes or oocytes injected with 5HT1A-R RNA alone. This current does not appear to represent the "basal" activity of the same channel activated by 5HT or ACh because it has a much lower sensitivity to  $Ba^{2+}$  block. Moreover, the fractionation data indicates that the RNA directing the expression of  $I_{hK}$  is smaller than that encoding the KG channel. However, it is not clear whether this atrial (or ventricle) RNA encodes the channel itself or a factor that enhances the expression or the activity of a native channel. Further studies, such as expression cloning, will help to identify the messages encoding the two inward rectifiers whose expression is reported here.

### Example 2



## Formation of Heteromultimeric Kir3.0 Channels

### Materials and methods

*Plasmids and DNAs.* To isolate Kir3.2 and Kir3.3 from mouse brain RNA, oligonucleotides were designed that anneal to the first assigned methionine codon and the assigned stop codon of mouse Kir3.2 and Kir3.3 (Lesage *et al* 5 (1994) FEBS Lett. **353**:37-42). Kir3.0-specific sequences were coupled to a 5'-end untranslated sequence from alfalfa mosaic virus (Jobling and Gehrke (1987) Nature **325**:622-625) and a T7 RNA polymerase recognition site to confer an optimal translational initiation site and allow RNA synthesis *in vivo* 10 (cRNA). At the 3' end, a poly(A) tail was added to confer RNA stability. Total RNA from adult mouse brain (purchased from Clontech) was used as template for cDNA synthesis (Sambrook *et al.*, *supra.*). PCR was conducted in a thermal cyclor (Perkin-Elmer) by using Vent Polymerase (New England Biolabs), which minimizes misincorporations during the amplification step. Kir3.2 and Kir3.3 15 PCR-derived sequences were then inserted into the pNoTA vector (5 Prime → 3 Primer, Inc.) for subsequent sequence analysis.

Kir3.1 cloned from cardiac atrium and inserted originally in pBluescript (Stratagene) was transferred to the vector pMXT (gift from J. Yang; University of Texas, Dallas), in which the cloning site is flanked by 5' and 3' untranslated 20 sequences from *Xenopus* globin. G protein subunits G $\beta$ 1 and G $\gamma$ 2 cDNAs were in the pFroggy vector (gift from L. Jan, University of California, San Francisco), as described in Lim *et al.* (1995) J. Gen. Physiol. **105**:421-439. The m2R cDNA (gift from E. Peralta, Harvard University) was in the pGEM3 vector (Promega).

*RNA Synthesis and Oocyte Injections.* Kir3.2 and Kir3.3 cRNAs were 25 synthesized directly from gel-isolated PCR products, while the remaining cRNAs were synthesized from linearized plasmid DNAs. cRNAs were dissolved in sterile water and injected into stage V or VI *Xenopus* oocytes as described in Quick and Lester (1994) in *Ion Channels of Excitable Cells*, ed.

Narahashi (Academic, San Diego) pp.261-279. Oocytes were maintained in ND96 solution. Oocytes were assayed 2-5 days after injection.

*Electrophysiology.* Whole-cell currents from oocytes were measured by using an Axoclamp 2A or Geneclamp 500 amplifier (Axon Instruments, Foster City, CA) in the two-electrode, voltage-clamp configuration. Current and voltage electrodes were filled with 3 M KCl to yield resistances ranging from 0.5 to 1.5 MΩ. Recordings were started in an external solution (0 K<sup>+</sup>) containing 98 mM NaCl, 1 mM MgCl<sub>2</sub>, and 5 mM Hepes, pH 7.3. In high-K<sup>+</sup>-containing solutions, the NaCl was replaced either completely by 98 mM KCl (98 K<sup>+</sup>), or partially, by 20 mM KCl (20 K<sup>+</sup>).

Cell-attached recordings of single channels were recorded from *Xenopus* oocytes as described in Methfessel et al. (1986) Pflugers Arch. 407, 577-588. Pipette solutions contained 150 mM KCl, 1 mM CaCl<sub>2</sub>, and 5 mM Hepes, pH 7.3 with KOH. Bath solution contained 150 mM KCl, 1 mM MgCl<sub>2</sub>, 1 mM EGTA, and 5 mM Hepes, pH 7.3 with KOH. For single-channel analysis, the current traces were filtered at 2 kHz and sampled at 10 kHz. Current amplitude histograms and open-time durations were obtained by using FETCHAN and pSTAT from pCLAMP 6.0 (Axon Instruments). All recordings were performed at room temperature (≈22°C).

## RESULTS

Kir3.1 is abundantly expressed in cardiac atrium and brain. When heterologously expressed in *Xenopus* oocytes, Kir3.1 induces strong inwardly rectifying K<sup>+</sup> currents either with m2R activation or with coexpression of G-protein subunits G<sub>β</sub>1 and G<sub>γ</sub>2.

Because expression of Kir3.2 and Kir3.1 was consistently smaller than for other channels expressed in oocytes, the effects of Kir3.1 and Kir3.2 coexpression under conditions of maximal m2R activation [1 μM acetylcholine

(ACh)] were examined. With the coinjection of Kir3.1 and Kir3.2 (Kir3.1 + 2) cRNAs (0.5 ng each per oocyte), exposure to ACh in these oocytes led to development of large inward currents when the voltage was jumped from 0 mV to more negative values. By contrast, in oocytes injected with Kir3.2 or Kir3.1 cRNAs (1 ng), receptor activated currents were much smaller in amplitude, comparable to levels reported previously. On average, Kir3.1 + 2 currents [inward currents induced by ACh ( $I_{K,ACh}$ ) =  $-6.4 \pm 0.8 \mu A$ ; mean  $\pm$  SEM;  $n = 5$ ] were about 9-fold larger than Kir3.2 currents ( $I_{K,ACh} = -698 \pm 98$  nA;  $n = 7$ ) and 17-fold larger than Kir3.1 currents ( $I_{K,ACh} = -365 \pm 45$  nA;  $n=5$ ).

Kir3.1 expressed in oocytes shows distinctive gating kinetics, including slow phases of activation (several hundred ms) during a jump from 0 mV to more negative potentials. For a similar voltage jump, Kir3.2 expressed in oocytes showed kinetics that more closely resemble other strong inward rectifiers, with a prominent phase of inactivation. These differences may be governed at least partially by differences in the sequence of the P region. The coexpressed subunits showed a slow phase of activation, but the time course of this activation cannot be explained as a simple weighted sum of the waveforms for Kir3.1 alone and Kir3.2 alone. Thus, the relations for jumps to voltages between -60 and -140 mV were well described by two exponential components with nearly voltage-independent time constants; at -80 mV the time constant of the slower component was  $213 \pm 12$  ms ( $n = 4$ ), or less than half that for Kir3.1 (32). These kinetic differences indicate molecular interactions between the Kir3.1 and Kir3.2 channels.

The large enhancement of the agonist-evoked currents for coexpressed channels might be explained by effects on any component in the receptor-channel signaling pathway, including receptors, endogenous G proteins, or the channels themselves. To discriminate among these possibilities, the receptor was uncoupled from the channel by overexpression of G protein subunits G $\beta$ 1

and G $\gamma$ 2. Importantly, cells expressing G $\beta$ 1 $\gamma$ 2 and Kir3.2 showed persistent inwardly rectifying currents at amplitudes comparable or larger to those observed for m2R activation of Kir3.2. Therefore, Kir3.2, like Kir3.1, is activated by G $\beta$  $\gamma$ .

5        Cells coexpressing Kir3.2 and Kir3.1 (Kir3.1 + 2) responded to high-K<sup>+</sup> solution with much larger currents than did cells expressing only Kir3.1 or Kir3.2, consistent with the results obtained with m2R activation. On average, Kir3.1 + 2 currents ( $I_K = -4.3 \pm 1 \mu A$ ;  $n = 7$ ) were 14 times larger than Kir3.2 currents ( $I_K = -305 \pm 56 \text{ nA}$ ;  $n = 7$ ) and 40 times larger than Kir3.1 currents ( $I_K =$   
10     $-104 \pm 10 \text{ nA}$ ;  $n = 6$ ). Thus, these experiments demonstrate that the large

mutual potentiating effect of Kir3.1 + 2 is independent of the method of G-protein activation and argue that the effects are on the channels themselves.

An increase in the number of channels or modification of the intrinsic channel properties might account for this potentiation. Single-channel  
15    recordings of Kir3.1 + 2 in combination or of Kir3.2 alone were made to test these possibilities. In oocytes coinjected with cRNAs for Kir3.2, G $\beta$ 1, and G $\gamma$ 2, single-channel currents in the cell-attached configuration displayed features consistent with macroscopic measurements, *i.e.* with hyperpolarization of the unit conductance increased. No outward currents were detected at membrane  
20    potentials positive to the K<sup>+</sup> equilibrium potential ( $E_K$ ;  $\approx 0 \text{ mV}$ ). These channels openings had a mean slope conductance of  $30 \pm 2 \text{ pS}$  ( $n = 4$ ) over the range from  $-40$  to  $-100 \text{ mV}$ , significantly smaller than the value of  $39 \text{ pS}$  for Kir3.1 alone, and showed bursts of flicker activity. Mean open-time distribution could be described by a fast component of  $0.1 \text{ ms}$  (35% of the total number of events)  
25    and a main slower component of  $0.5 \text{ ms}$  (65% of the total number of events).

The combination of Kir3.1 + 2 produced unitary currents with strong inward rectification and a mean slope conductance of  $35 \pm 3 \text{ pS}$  ( $n = 4$ ), intermediate between the values for Kir3.1 alone and Kir3.2 alone. In addition,

Kir3.1 + 2 channels displayed markedly longer openings than Kir3.2 channels. Recordings from Kir3.1 + 2 channels showed a 7-fold increase in the major component of open-time duration (3.5 ms; 71% of the total number of events; there was also a smaller component of 0.5 ms; 29% of the total number of events) compared to Kir3.2. Qualitatively, the Kir3.1 + 2 patch recordings are rather similar to those observed for Kir3.1 in terms of mean open time, although the typically low expression levels for Kir3.1 alone vitiate systematic comparisons. At present, it is not known whether this increase, like the increase in macroscopic currents on Kir3.1 + 2 coexpression, is solely explained by the increase in open-time duration or whether the enhanced currents also arise from an increase in the number of openings, either because each channel opens more often or because there are more functional channels.

The effects of Kir3.3 coexpression was tested with Kir3.1 or Kir3.2. Injection of Kir3.3, G $\beta$ <sub>1</sub>, and G $\gamma$ <sub>2</sub> cRNAs did not activate inward currents in high-K<sup>+</sup>-containing solution; nor were currents activated by opioid receptors. Nevertheless, Kir3.3 had profound effects when coexpressed with Kir3.1. Coexpression of Kir3.1 and Kir3.3 (Kir3.1 + 3) resulted in 7-fold larger I<sub>K</sub> currents (I<sub>K</sub> = -3.4 ± 0.5 μA; n = 6) than for Kir3.1 alone (I<sub>K</sub> = -481 ± 43 nA; n = 6). Such augmentation was seen for each of several batches of oocytes and cRNAs and on activation of the m2R pathway. Thus, these results demonstrate that Kir3.3, despite not being directly activated by G protein subunits G $\beta$ <sub>1</sub> and G $\gamma$ <sub>2</sub>, can interact with, and increase, G-protein-mediated responses of Kir3.1.

Surprisingly, Kir3.3 had a suppressing effect when coexpressed with Kir3.2. G $\beta\gamma$ -induced, inwardly rectifying currents in oocytes coinjected with Kir3.2 and Kir3.3 cRNAs (0.5 ng each per oocyte) (I<sub>K</sub> = -129 ± 22 nA; n = 7) were much smaller than in oocytes injected with Kir3.2 cRNA alone (1 ng per

oocyte)  $I_K = -5.1 \pm 0.6 \mu A$ ;  $n = 9$ ). This dominant-negative effect of GIRk3 on Kir3.2 currents was also observed when m2R was coexpressed, instead of G-protein subunits  $G\beta_1$  and  $G\gamma_2$ .

A very important issue is raised by the occurrence of heteromultimeric channels is their differential distribution in excitable tissues and their physiological significance. Distinct Kir3.0 heteromultimeric channels may be modulated or activated by G proteins differentially, as reported for other G protein effectors. This allows for a larger degree of flexibility in the control of this important form of neuronal signaling.

The plasmid pBSIIKS(-)KGA, encoding the rat inward rectifier, G-protein activated, mammalian, potassium KGA channel was deposited on May 17, 1993 with the American Type Culture Collection (ATCC), 12301 Parklawn Drive, Rockville, Maryland 20852, U.S.A., under the provisions of the Budapest Treaty for the International Recognition of the Deposition of Microorganism for the Purposes of Patent Procedure, and given the ATCC accession number 75469.

All publications and patent applications cited in this specification are herein incorporated by reference as if each individual publication or patent application were specifically and individually indicated to be incorporated by reference.

Although the foregoing invention has been described in some detail by way of illustration and example for purposes of clarity of understanding, it will be readily apparent to those of ordinary skill in the art in light of the teachings of this invention that certain changes and modifications may be made thereto without departing from the spirit or scope of the appended claims.

WHAT IS CLAIMED IS:

1. An isolated DNA molecule encoding at least two different inward rectifier, G-protein activated, mammalian, potassium Kir3.0 polypeptides, wherein said nucleic acid is characterized by its ability to cause a change in potassium flow across a *Xenopus* oocyte cell membrane upon expression therein.
2. A nucleic acid according to Claim 1, wherein said mammalian Kir3.0 polypeptides are Kir3.1/KGA and Kir3.2.
3. A nucleic acid according to Claim 1, wherein said mammalian Kir3.0 polypeptides are Kir3.1/KGA and Kir3.3.
4. A nucleic acid according to Claim 1, wherein said mammalian Kir3.0 polypeptides are Kir3.2 and Kir3.3.
5. A nucleic acid according to Claim 2, wherein said Kir3.1/KGA polypeptide has the amino acid sequence of SEQ ID NO:2.
6. A method of producing a functional Kir3.0 channel in an expression host cell, the method comprising:  
introducing into said expression host cell a nucleic acid encoding a first mammalian Kir3.0 polypeptide and a nucleic acid encoding a second mammalian Kir3.0 polypeptide into said heterologous cell under conditions that permit expression of said nucleic acids;  
wherein said mammalian Kir3.0 polypeptides assemble to form a functional Kir3.0 in said expression host cell.

7. A method according to Claim 6, wherein said nucleic acid encoding said first mammalian Kir3.0 polypeptide and said nucleic acid encoding said second mammalian Kir3.0 polypeptide are present on a single vector.

5

8. A method according to Claim 6, wherein said nucleic acid encoding said first mammalian Kir3.0 polypeptide and said nucleic acid encoding said second mammalian Kir3.0 polypeptide are present on different vectors.

10

9. A method according to Claim 6, wherein said mammalian Kir3.0 polypeptides are Kir3.1/KGA and Kir3.2.

10. A method according to Claim 6, wherein said mammalian Kir3.0 polypeptides are Kir3.1/KGA and Kir3.3.

15

11. A method according to Claim 6, wherein said mammalian Kir3.0 polypeptides are Kir3.2 and Kir3.3.

20

12. A host cell comprising a functional heterologous Kir3.0 channel; wherein said functional Kir3.0 channel comprises a first Kir3.0 polypeptide and a second mammalian Kir3.0 polypeptide assembled to form a functional Kir3.0 channel.

25

13. A host cell according to Claim 12, wherein said mammalian Kir3.0 polypeptides are Kir3.1/KGA and Kir3.2.



14. A host cell according to Claim 12, wherein said mammalian Kir3.0 polypeptides are Kir3.1/KGA and Kir3.3.

15. A host cell according to Claim 12, wherein said mammalian  
5 Kir3.0 polypeptides are Kir3.2 and Kir3.3.

16. A host cell according to Claim 12, wherein said host cell is a *Xenopus laevis* oocyte.

10            17. A method of screening for agents that modulate the activity of a Kir3.0 channel, the method comprising:

              combining a candidate agent with a functional Kir3.0 channel under conditions that permit inward K<sup>+</sup> current

              determining the induced current;

15            wherein a change in said induced current in the presence of said agent as compared to a control is indicative that said agent modulates the activity of a Kir3.0 channel.

INWARD RECTIFIER, G-PROTEIN ACTIVATED, MAMMALIAN, POTASSIUM  
CHANNELS AND USES THEREOF

5

## ABSTRACT OF THE DISCLOSURE

Compositions and methods are provided for producing functional mammalian inward rectifier, G-protein activated potassium channels (Kir3.0 channels). A channel is a multimeric protein comprising one or more Kir3.0 polypeptides, e.g. Kir3.1, Kir3.2, etc., where the polypeptides may be from the same or different species. The functional channel has the distinctive features of an anomalous rectifier, in that it conducts inward but not outward  $K^+$  current; it is blocked by low concentrations of extracellular  $Cs^+$  or  $Ba^{2+}$ ; and the conductance of the channel does not depend solely on voltage, but on  $(E-E_K)$ . The ability of the channel to conduct inward  $K^+$  current is modulated by G-proteins, particularly G-proteins of the  $G_i/G_o$  family. A number of mammalian cell surface receptors activate G-proteins as a consequence of specific ligand binding. The signal transduction from receptor to Kir3.0 channel is therefore coupled through G-protein intermediates.

The functional Kir3.0 channels are useful in drug screening assays directed to modulation of cellular electrophysiology. Nucleic acids encoding Kir3.0 polypeptides are useful for expression of the gene product, and for identification of homologous genes from other species, as well as other members of the same family of proteins. Expression of the nucleic acids in a heterologous cell, e.g. *Xenopus* oocyte, confers the ability to cause a change in potassium flow in response to G-protein activation.

# SEQUENCE LISTING

## (1) GENERAL INFORMATION:

- (i) APPLICANTS: Henry A. Lester, Norman Davidson, Paulo Kofuji
- (ii) TITLE OF INVENTION: Inward Rectifier, G-Protein Activated, Mammalian, Potassium Channels and Uses Thereof
- (iii) NUMBER OF SEQUENCES: 2
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  - (F) ZIP: 94111-4187
- (v) COMPUTER READABLE FORM:
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  - (B) COMPUTER: IBM PC compatible
  - (C) OPERATING SYSTEM: PC-DOS/MS-DOS
  - (D) SOFTWARE: PatentIn Release #1.0, Version #1.25
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  - (A) APPLICATION NUMBER: US
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## (2) INFORMATION FOR SEQ ID NO:1:

- (i) SEQUENCE CHARACTERISTICS:
    - (A) LENGTH: 2076 base pairs
    - (B) TYPE: nucleic acid
    - (C) STRANDEDNESS: single
    - (D) TOPOLOGY: linear
  - (ii) MOLECULE TYPE: cDNA
  - (ix) FEATURE:
    - (A) NAME/KEY: CDS
    - (B) LOCATION: 32..1534
  - (xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:
- |   |     |
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| Met Ser Ala Leu Arg Arg Lys                                     |     |
| 1 5   |     |
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| Phe Gly Asp Asp Tyr Gln Val Val Thr Thr Ser Ser Ser Gly Ser Gly |     |
| 10 15 20  |     |
| TTG CAG CCC CAG GGG CCA GGA CAG GGC CCA CAG CAG CAG CTT GTA CCC | 148 |
| Leu Gln Pro Gln Gly Pro Gly Gln Gly Pro Gln Gln Gln Leu Val Pro |     |
| 25 30 35  |     |

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CAG CAC GGC AAC CTG GGC AGC GAG ACC AGT CGC TAC CTT TCC GAC CTC Gln His Gly Asn Leu Gly Ser Glu Thr Ser Arg Tyr Leu Ser Asp Leu 60 65 70	244
TTC ACT ACC CTG GTG GAT CTC AAG TGG CGT TGG AAC CTC TTT ATC TTC Phe Thr Thr Leu Val Asp Leu Lys Trp Arg Trp Asn Leu Phe Ile Phe 75 80 85	292
ATC CTC ACC TAC ACC GTG GCC TGG CTC TTC ATG GCG TCC ATG TGG TGG Ile Leu Thr Tyr Thr Val Ala Trp Leu Phe Met Ala Ser Met Trp Trp 90 95 100	340
GTG ATC GCT TAT ACC CGG GGC GAC CTG AAC AAA GCC CAT GTC GGC AAC Val Ile Ala Tyr Thr Arg Gly Asp Leu Asn Lys Ala His Val Gly Asn 105 110 115	388
TAC ACT CCC TGT GTG GCC AAT GTC TAT AAC TTC CCC TCT GCC TTC CTT Tyr Thr Pro Cys Val Ala Asn Val Tyr Asn Phe Pro Ser Ala Phe Leu 120 125 130 135	436
TTC TTC ATC GAG ACC GAG GCC ACC ATC GGC TAT GGC TAC CGC TAC ATC Phe Phe Ile Glu Thr Glu Ala Thr Ile Gly Tyr Gly Tyr Arg Tyr Ile 140 145 150	484
ACC GAC AAG TGC CCC GAG GGC ATC ATC CTT TTC CTT TTC CAG TCC ATC Thr Asp Lys Cys Pro Glu Gly Ile Ile Leu Phe Leu Phe Gln Ser Ile 155 160 165	532
CTT GGC TCC ATC GTG GAC GCT TTC CTC ATC GGC TGC ATG TTC ATC AAG Leu Gly Ser Ile Val Asp Ala Phe Leu Ile Gly Cys Met Phe Ile Lys 170 175 180	580
ATG TCC CAG CCC AAA AAG CGC GCC GAG ACC CTC ATG TTT AGC GAG CAT Met Ser Gln Pro Lys Lys Arg Ala Glu Thr Leu Met Phe Ser Glu His 185 190 195	628
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CTT GTG TCC CCT CTC ACC ATT TGC CAC GTG ATC GAT GCC AAA AGC CCC Leu Val Ser Pro Leu Thr Ile Cys His Val Ile Asp Ala Lys Ser Pro 265 270 275	868
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GTC GTC ATC CTG GAA GGC ATC GTG GAA ACC ACA GGG ATG ACT TGT CAA Val Val Ile Leu Glu Gly Ile Val Glu Thr Thr Gly Met Thr Cys Gln 300 305 310	964

GCT	CGA	ACA	TCA	TAC	ACC	GAA	GAT	GAA	GTT	CTT	TGG	GGT	CAT	CGT	TTT		1012
Ala	Arg	Thr	Ser	Tyr	Thr	Glu	Asp	Glu	Val	Leu	Trp	Gly	His	Arg	Phe		
			315					320					325				
TTC	CCT	GTA	ATT	TCT	TTA	GAA	GAA	GGA	TTC	TTT	AAA	GTC	GAT	TAC	TCC		1060
Phe	Pro	Val	Ile	Ser	Leu	Glu	Glu	Gly	Phe	Phe	Lys	Val	Asp	Tyr	Ser		
		330					335					340					
CAG	TTC	CAT	GCA	ACC	TTT	GAA	GTC	CCC	ACC	CCT	CCG	TAC	AGT	GTG	AAA		1108
Gln	Phe	His	Ala	Thr	Phe	Glu	Val	Pro	Thr	Pro	Pro	Tyr	Ser	Val	Lys		
		345				350					355						
GAG	CAG	GAA	GAA	ATG	CTT	CTC	ATG	TCT	TCC	CCT	TTA	ATA	GCA	CCA	GCC		1156
Glu	Gln	Glu	Glu	Met	Leu	Leu	Met	Ser	Ser	Pro	Leu	Ile	Ala	Pro	Ala		
360					365					370					375		
ATA	ACC	AAC	AGC	AAA	GAA	AGA	CAC	AAT	TCT	GTG	GAG	TGC	TTA	GAT	GGA		1204
Ile	Thr	Asn	Ser	Lys	Glu	Arg	His	Asn	Ser	Val	Glu	Cys	Leu	Asp	Gly		
				380					385					390			
CTA	GAT	GAC	ATT	AGC	ACA	AAA	CTT	CCA	TCG	AAG	CTG	CAG	AAA	ATT	ACG		1252
Leu	Asp	Asp	Ile	Ser	Thr	Lys	Leu	Pro	Ser	Lys	Leu	Gln	Lys	Ile	Thr		
			395					400					405				
GGG	AGA	GAA	GAC	TTT	CCC	AAA	AAA	CTC	CTG	AGG	ATG	AGT	TCT	ACA	ACT		1300
Gly	Arg	Glu	Asp	Phe	Pro	Lys	Lys	Leu	Leu	Arg	Met	Ser	Ser	Thr	Thr		
		410					415					420					
TCA	GAA	AAA	GCC	TAT	AGT	TTG	GGT	GAT	TTG	CCC	ATG	AAA	CTC	CAA	CGA		1348
Ser	Glu	Lys	Ala	Tyr	Ser	Leu	Gly	Asp	Leu	Pro	Met	Lys	Leu	Gln	Arg		
		425				430					435						
ATA	AGT	TCG	GTT	CCT	GGC	AAC	TCT	GAA	GAA	AAA	CTG	GTA	TCT	AAA	ACC		1396
Ile	Ser	Ser	Val	Pro	Gly	Asn	Ser	Glu	Glu	Lys	Leu	Val	Ser	Lys	Thr		
440					445					450					455		
ACC	AAG	ATG	TTA	TCA	GAT	CCC	ATG	AGC	CAG	TCT	GTG	GCC	GAT	TTG	CCA		1444
Thr	Lys	Met	Leu	Ser	Asp	Pro	Met	Ser	Gln	Ser	Val	Ala	Asp	Leu	Pro		
				460					465					470			
CCG	AAG	CTT	CAA	AAG	ATG	GCT	GGA	GGA	CCT	ACC	AGG	ATG	GAA	GGG	AAT		1492
Pro	Lys	Leu	Gln	Lys	Met	Ala	Gly	Gly	Pro	Thr	Arg	Met	Glu	Gly	Asn		
			475				480					485					
CTT	CCA	GCC	AAA	CTA	AGA	AAA	ATG	AAC	TCT	GAC	CGC	TTC	ACA				1534
Leu	Pro	Ala	Lys	Leu	Arg	Lys	Met	Asn	Ser	Asp	Arg	Phe	Thr				
		490					495					500					
TAGCAAAACA CCCCATTAGG CATTATTTCA TGTTTTGATT TAGTTTTAGT CCAATATTTG																	1594
GCTGATAAGA TAATCCTCCC CGGGAAATCT GAGAGGTCTA TCCCAGTCTG GCAAATTCAT																	



[illegible][illegible]

Figure 1 shows a graph of current (I) versus voltage (V). The x-axis is labeled with voltage values: -70, -60, -50, -40, -30, -20, -10, 0, 10, 20, 30, 40, 50, 60, 70, 80, 90, 100. The y-axis is labeled with current values: -100, -200, -300, -400, -500, -600, -700, -800, -900, -1000, -1100, -1200, -1300, -1400, -1500, -1600, -1700, -1800, -1900, -2000, -2100, -2200, -2300, -2400, -2500, -2600, -2700, -2800, -2900, -3000, -3100, -3200, -3300, -3400, -3500, -3600, -3700, -3800, -3900, -4000, -4100, -4200, -4300, -4400, -4500, -4600, -4700, -4800, -4900, -5000, -5100, -5200, -5300, -5400, -5500, -5600, -5700, -5800, -5900, -6000, -6100, -6200, -6300, -6400, -6500, -6600, -6700, -6800, -6900, -7000, -7100, -7200, -7300, -7400, -7500, -7600, -7700, -7800, -7900, -8000, -8100, -8200, -8300, -8400, -8500, -8600, -8700, -8800, -8900, -9000, -9100, -9200, -9300, -9400, -9500, -9600, -9700, -9800, -9900, -10000. The graph shows a baseline current-voltage relationship (solid line) and a response to 4 nM 5HT (dashed line). The current is inhibited by 5HT, with the inhibition being more pronounced at more negative voltages. The inhibition is labeled  $I_{hK}$  and  $I_{5HT}$ . The graph is labeled ND96, HIGH  $K^+$ , and 4nM 5HT. Scale bars indicate 100 nA and 1 min.

FIGURE 1B

10  $\mu$ M AcCho      0.2  $\mu$ M 5HT

100 nA  
1 min

The figure displays two electrophysiological traces. The left trace, labeled '10  $\mu$ M AcCho', shows a baseline membrane potential that dips slightly and then returns to baseline. The right trace, labeled '0.2  $\mu$ M 5HT', shows a baseline membrane potential that drops sharply and remains at a lower level. A scale bar at the bottom indicates 100 nA for the vertical axis and 1 min for the horizontal axis.

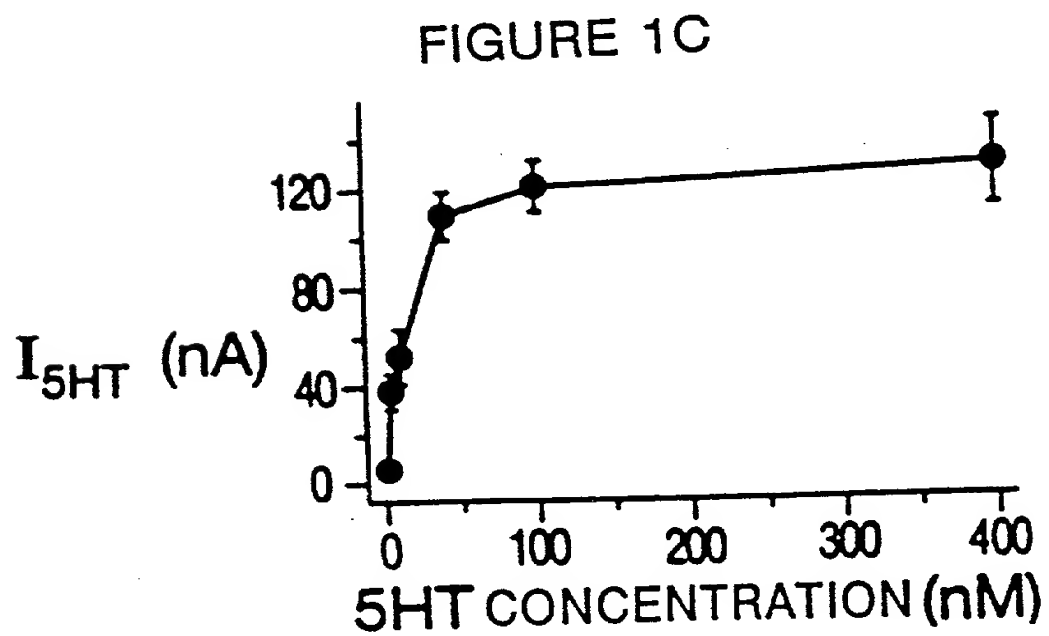




FIGURE 2A

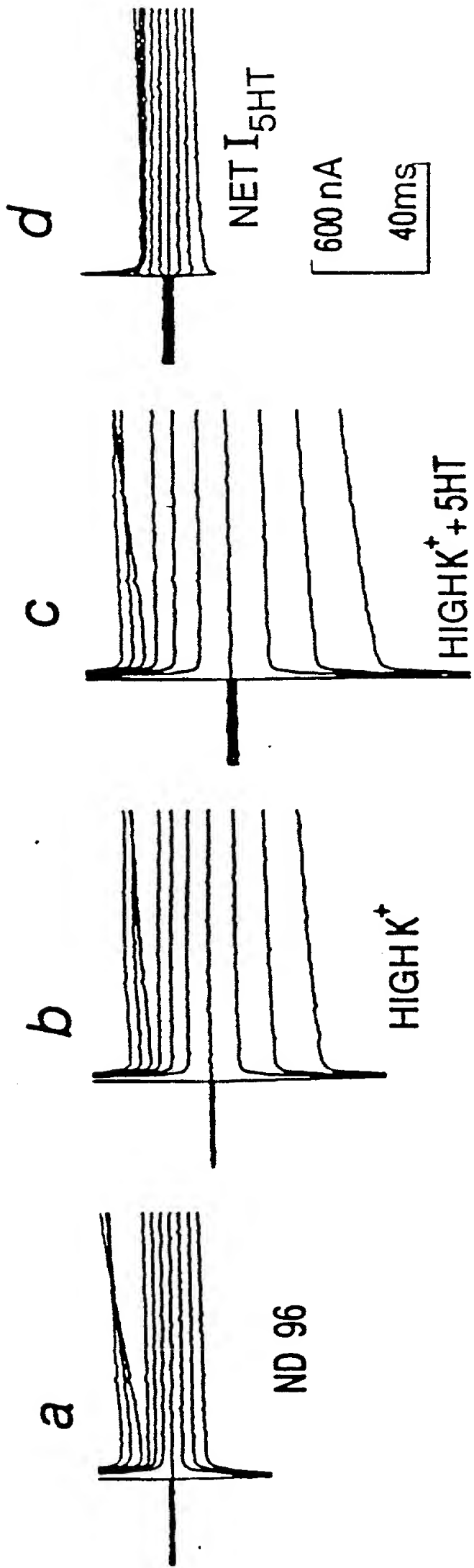


FIGURE 2B

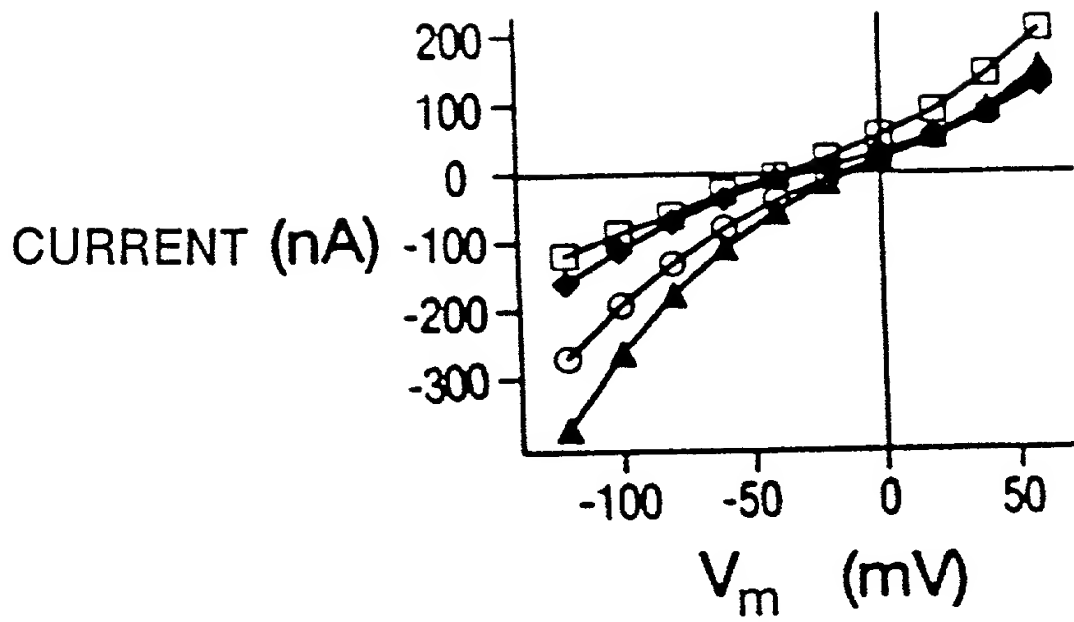


FIGURE 2C

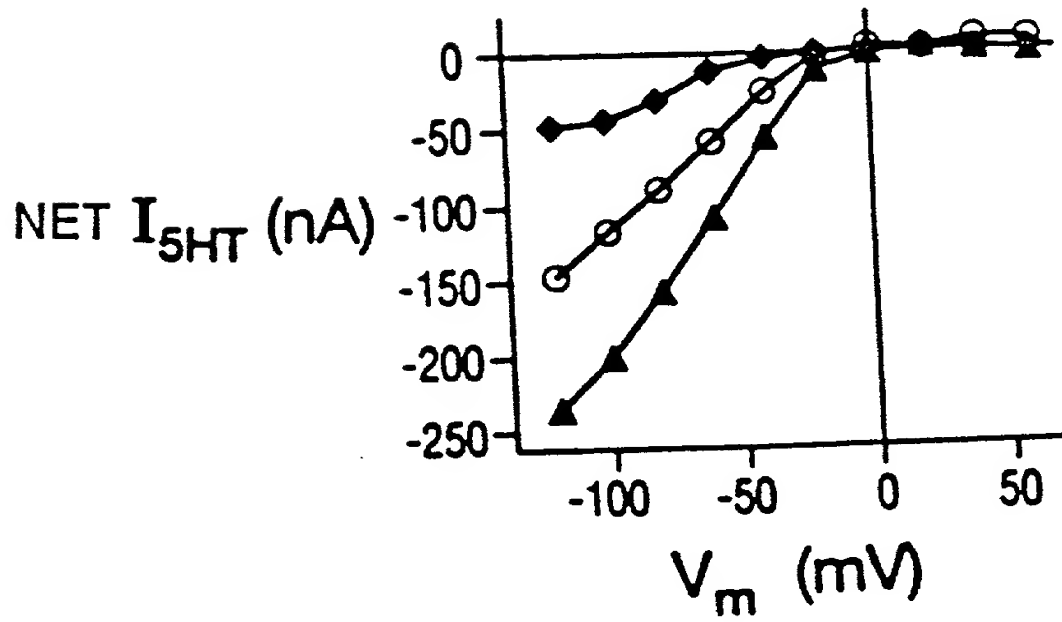


FIGURE 2D

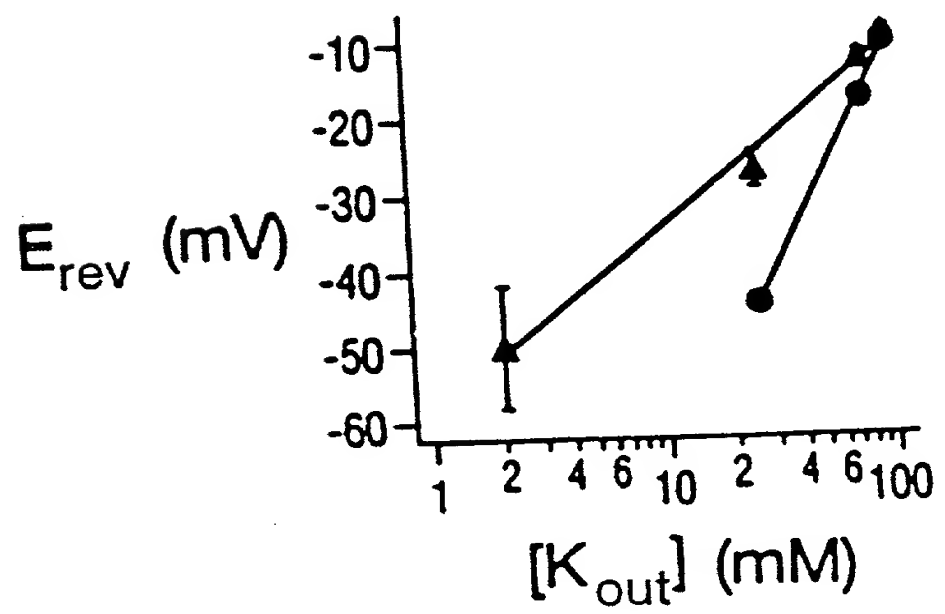


FIGURE 3A

FIGURE 3B

FIGURE 3C

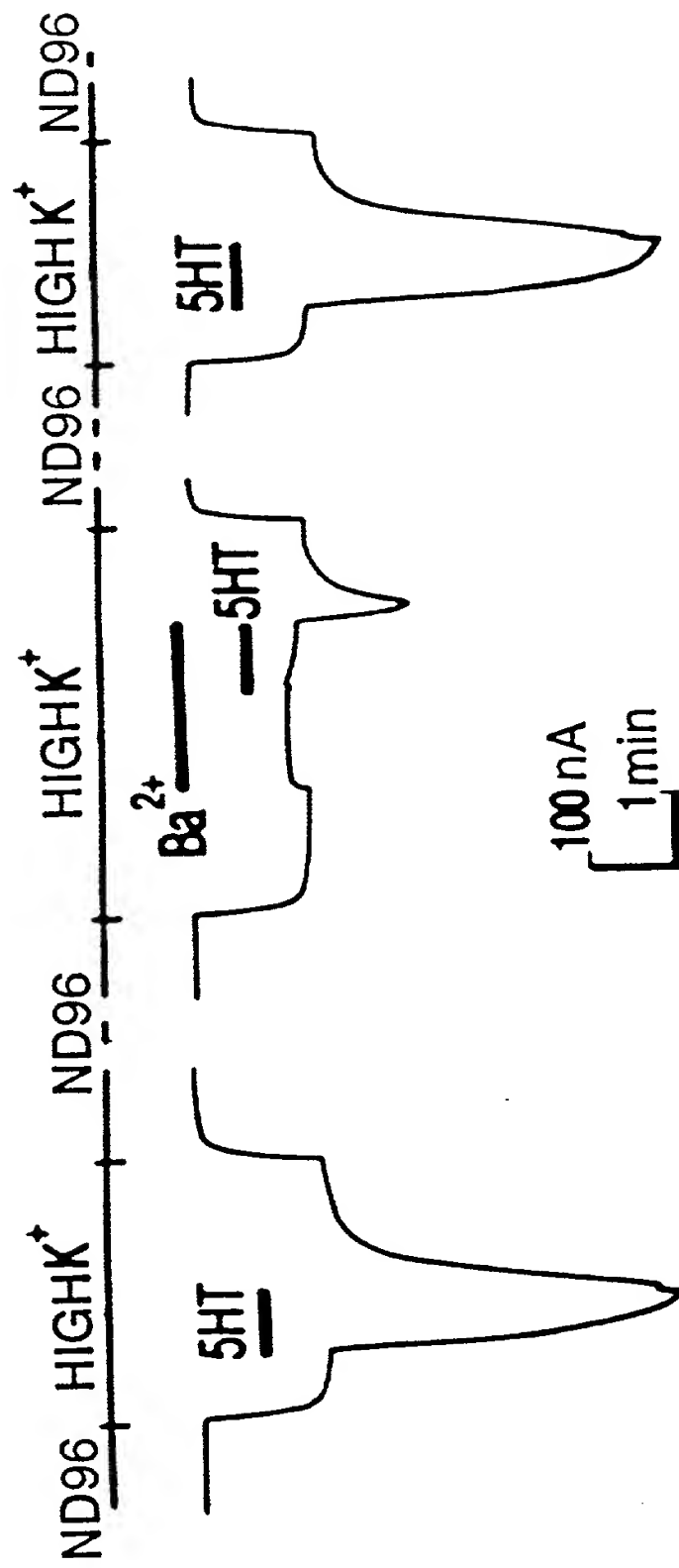


Figure 1 is a line graph showing the percentage of control current versus the concentration of  $\text{Ba}^{2+}$  (mM) for three different current components:  $I_{hK}$  (native),  $I_{hK}$  (atrium), and  $I_{5HT}$ . The y-axis is labeled '% OF CONTROL CURRENT' and ranges from 0 to 100. The x-axis is labeled ' $[\text{Ba}^{2+}]$  (mM)' and is on a logarithmic scale from 0.01 to 10.  $I_{hK}$  (native) is represented by filled circles,  $I_{hK}$  (atrium) by open circles, and  $I_{5HT}$  by open triangles. All three currents show a decrease in percentage of control current as  $\text{Ba}^{2+}$  concentration increases.  $I_{5HT}$  shows the most rapid and significant inhibition, dropping to near zero by 10 mM.  $I_{hK}$  (native) and  $I_{hK}$  (atrium) show a more gradual, similar inhibition.

$[\text{Ba}^{2+}]$ (mM)	$I_{hK}$ (native) (%)	$I_{hK}$ (atrium) (%)	$I_{5HT}$ (%)
0.01	100	100	80
0.03	95	98	62
0.1	85	92	35
0.3	78	85	15
1.0	75	75	5
3.0	68	68	0
10.0	38	55	0

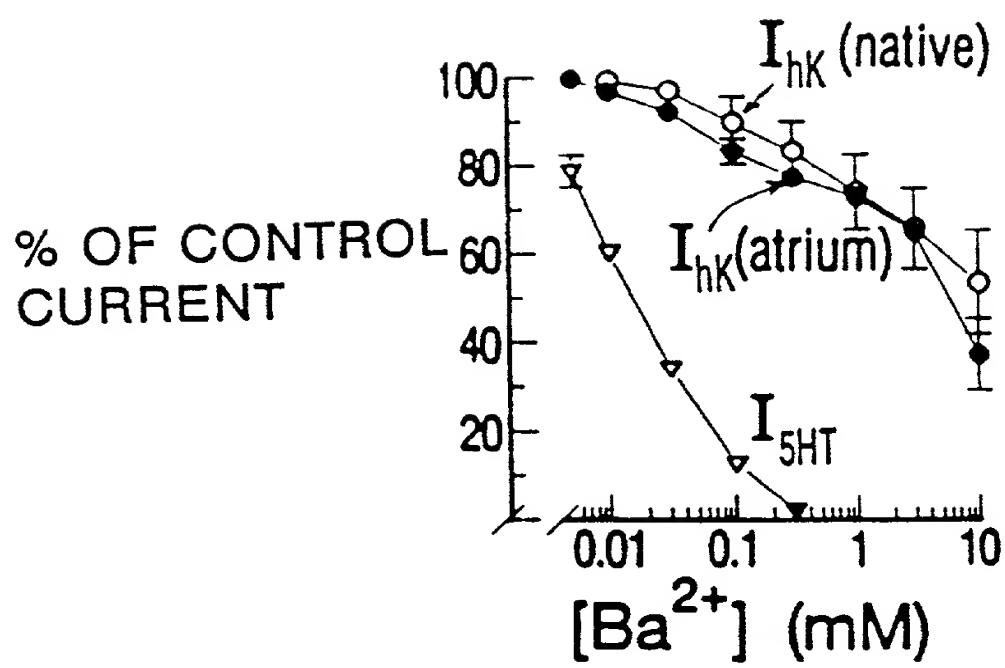


FIGURE 4A

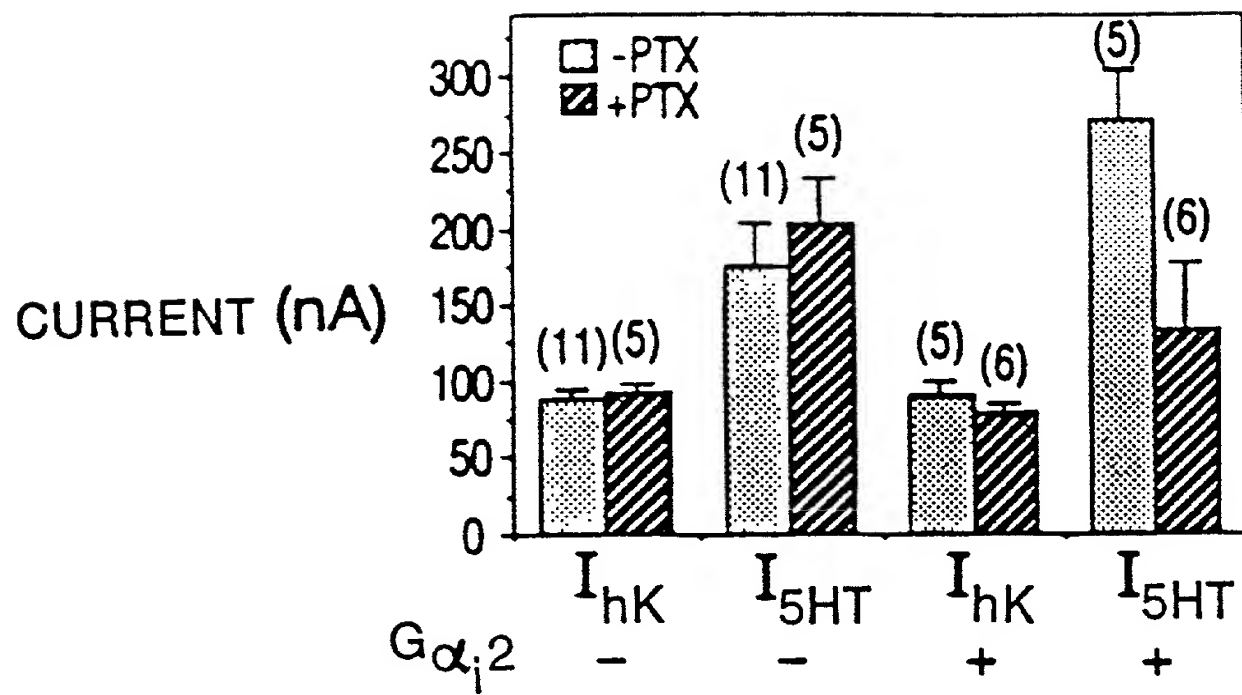
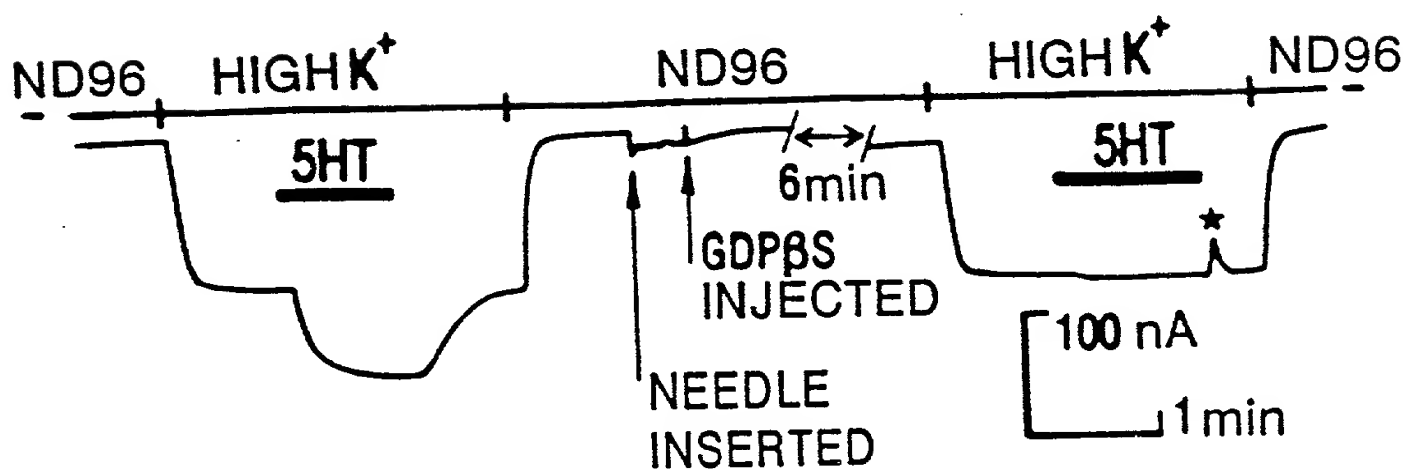


FIGURE 4B



## DECLARATION FOR PATENT APPLICATION

As a below-named inventor, I hereby declare that:

My residence, post office address and citizenship are as stated below next to my name,

I believe I am the original, first and sole inventor (if only one name is listed below) or an original, first and joint inventor (if plural names are listed below) of the subject matter which is claimed and for which a patent is sought on the invention entitled INWARD RECTIFIER, G-PROTEIN ACTIVATED, MAMMALIAN, POTASSIUM CHANNELS AND USES THEREOF, the specification of which

(check ☐ is attached hereto.  
one)

☒ was filed on 7 March 1996 as  
Application Serial No. \_\_\_\_\_  
and was amended on \_\_\_\_\_  
(if applicable)

I hereby state that I have reviewed and understand the contents of the above-identified specification, including the claims, as amended by any amendment referred to above.

I acknowledge the duty to disclose to the Patent Office all information known to me to be material to patentability as defined in 37 C.F.R. 1.56.

I hereby claim foreign priority benefits under Title 35, United States Code, §119 of any foreign application(s) for patent or inventor's certificate listed below and have also identified below any foreign application for patent or inventor's certificate having a filing date before that of the application on which priority is claimed:

Prior Foreign Application(s)			Priority Claimed	
_____	_____	_____	<input type="checkbox"/>	<input type="checkbox"/>
(Number)	(Country)	(Day/Month/Year Filed)	Yes	No
_____	_____	_____	<input type="checkbox"/>	<input type="checkbox"/>
(Number)	(Country)	(Day/Month/Year Filed)	Yes	No
_____	_____	_____	<input type="checkbox"/>	<input type="checkbox"/>
(Number)	(Country)	(Day/Month/Year Filed)	Yes	No

I hereby claim the benefit under Title 35, United States Code, §120 of any United States application(s) listed below and, insofar as the subject matter of each of the claims of this application is not disclosed in the prior United States application in the manner provided by the first paragraph of Title 35, United States Code, §112, I acknowledge the duty to disclose to the Patent Office all information known to me to be material to patentability as defined in 37 C.F.R. 1.56 which occurred between the filing date of the prior application and the national or PCT international filing date of this application:

<u>08/066,371</u>	<u>21 March 1993</u>	<u>Pending</u>
(Application Serial No.)	(Filing Date)	(Status)
		(patented, pending, abandoned)

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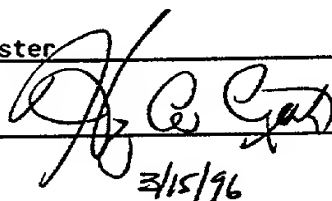
File No. A-63098/RFT/PJS

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Title 18, United States Code, §1001 and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

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0903997-034699